Chapter 3

Cigarette smoke enhances resistance to apoptosis; relevance for its opposite effects in inflammatory bowel diseases

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ABSTRACT

BACKGROUND & AIMS
Cigarette smoking is associated with inflammatory bowel diseases (IBD), but with a remarkable opposite effect in Crohn’s disease (CD) and ulcerative colitis (UC). Smoking aggravates CD and ameliorates UC. Here, we investigated the smoke-induced signaling pathways in order to explain this dichotomy.

METHODS
Intestinal epithelial cells (DLD-1) and T-cells (Jurkat) were exposed to cigarette smoke extract (CSE) and cellular changes in signaling pathways were identified using kinome profiling. Cells were exposed to inflammatory triggers (anti-Fas, cytokines, menadione) in presence or absence of CSE, nicotine, acrolein or carbon monoxide-releasing molecules (CORMs). Apoptosis was quantified by caspase-3 assays and PARP cleavage. Contribution of apoptotic pathways was studied using specific inhibitors. Necrosis and cell viability were determined by Sytox Green nuclear staining and WST-1 assays, respectively.

RESULTS
Kinome analyses revealed that the canonical apoptotic pathway was inhibited by CSE, while multiple survival pathways were stimulated simultaneously, including AMPK, PI3K and ERK1/2. CSE dose-dependently (from 0-20% CSE) suppressed caspase-3 activity provoked by anti-Fas, cytokine mixture or menadione in both DLD-1 and Jurkat cells with a concomitant increase in cellular metabolic activity. CSE concentrations above 30% induced necrosis. Inhibition of individual CSE-activated anti-apoptotic signaling partially reversed CSE effects on survival. All tested cigarette smoke compounds showed anti-apoptotic effects, with CORMs being the most potent.

CONCLUSION
Smoke components exert multiple anti-apoptotic effects in epithelial cells and T-lymphocytes, which may aggravate CD that is characterized by apoptosis-resistant T-cells. Conversely, it may prevent excessive damage to the colonic epithelium and thereby ameliorate UC.
INTRODUCTION

Crohn’s disease (CD) and ulcerative colitis (UC) are the two major types of inflammatory bowel disease (IBD). CD is characterized by transmural discontinuous inflammation with granuloma and may affect the whole gastrointestinal tract. UC is characterized by a superficial continuous inflammation of the mucosa of the colon ascending from the rectum. Both CD and UC develop as a consequence of a complex interplay between genetic and environmental factors. Recent genome-wide association studies have provided a comprehensive map of genes that are linked to the propensity to these diseases. Though instrumental for defining the cellular processes that may promote CD or UC, genetic predisposition only explains about 20% of CD and <10% of UC disease development. This implies that IBD is primarily caused by environmental factors. The most prominent environmental factor that is implicated in the development of IBD is cigarette smoke and was first documented in the early 1980s, with smokers being overrepresented in CD and underrepresented in UC compared to the healthy control population. Since then, many studies have confirmed that smoking aggravates CD and ameliorates UC. Smoking is associated with a higher prevalence of ileal disease in CD, more complex disease, increased need for steroids and immunosuppressive therapy as well as poorer response to therapy with infliximab. In contrast, smoking is associated with less hospitalizations in UC and decreased need for steroids and surgery. Recently, we found that also passive smoking is detrimental for CD patients and that active smoking showed dose-dependent beneficial effects in UC.

How smoking modulates disease course and therapeutic outcome in IBD is still unclear. Several potential mechanisms, including smoke-provoked alterations in mucosal immune responses, modifications in intestinal mediators and cytokines and modulation of gut permeability have been proposed, but none of these offers a satisfying explanation for the dichotomal effects of smoking on CD and UC. The intestinal tract is composed of several barriers to prevent pathogenic invasion. However, bacteria and dietary antigens constantly pass the epithelium leading to a state of controlled inflammation. Disruption of the barrier allows luminal pathogens to pass the epithelium and ongoing exposure of pathogens triggers the immune system and causes inflammation of the colon leading to epithelial damage. To limit inflammation to a subclinical state, a delicate balance between continuous cell replacement and repair of the epithelium is required. Regulatory mechanisms are essential for eliminating damaged cells during tissue remodeling and inflammation, processes that appear to be modulated by cigarette smoke with opposite outcomes.
in CD and UC. Therefore, we set out to identify the regulatory mechanisms provoked by cigarette smoke and studied their putative role in the dichotomous effects on the two main forms of IBD. Defining the cigarette smoke-induced cellular (dys)functions will help to understand disease progression in smoking and non-smoking patients with CD or UC and may unveil novel therapeutic targets for these diseases.

MATERIALS AND METHODS

CELL LINES AND CULTURE CONDITIONS

Human colonic epithelial cell lines (DLD-1, SW480, HCT116, Caco-2) and T-lymphocyte cell lines (Jurkat, MOLT-4) were cultured in T75 flasks (Greiner bio-one, Alphen aan den Rijn, The Netherlands) as described previously. Cells were exposed to various apoptotic stimuli in presence or absence of different concentrations cigarette smoke extract (CSE; 0-100% v/v) as specified in the result section. CSE was prepared as described by passing smoke from 2 standard 3R4F research cigarettes (Lexington, KY, USA) through 25 ml culture medium, which was defined as 100% CSE. Death receptor-mediated apoptosis was induced by anti-Fas (1 µg/ml) or cytokine mixture (CM; consisting of TNF-α, IFN-γ and IL-1β, 10 ng/ml each). Oxidative stress-mediated apoptosis was induced by the superoxide anion donor menadione (50 mmol/l). Anti-Fas-induced caspase-3 activity is maximal at 9 and 3 hours in DLD-1 and Jurkat cells, respectively. For CM- and menadione-induced apoptosis, caspase-3 activity peaks at 16 hours in DLD-1 cells. Therefore, these time-points were chosen to study the effect of CSE.

In addition, cells were exposed to a water-soluble fraction of CSE, which was obtained by a “water pipe” setting. Smoke from 2 cigarettes was first passed through 25 ml HBSS-buffer (= 100% water pipe medium) and subsequently led through 25 ml culture medium (= 100% CSE after water pipe medium). Furthermore, cells were exposed to various pure compounds of cigarette smoke, e.g. nicotine, acrolein and carbon monoxide (CO), to study their effect on anti-Fas-, CM-, or menadione-induced apoptosis. Nicotine (#N5260; Sigma-Aldrich, MO, USA) was added at concentrations 0.1-10 µM. Acrolein (#A1679; Sigma) was used at concentrations of 25-100 µM. CO was either diffused through 25 ml medium, similar as preparing CSE, or by adding CO-releasing molecule-2 (CORM-2, tricarbonyldichlororuthenium II dimer, #288144; Sigma) at concentrations of 50-200 µM. In parallel, cells were incubated with inactivated (incubated at 37°C for 24 hour and saturated with nitrogen for 1 hour just before use) CORM-2.
KINOME PROFILING AND DATA ACQUISITION

The peptide array data analysis was done as described earlier\(^14\). Spot density and background from three technical replicates of each individual experimental sample were analyzed using Scanalyze (http://rana.lbl.gov/EisenSoftware). For each peptide the average and standard deviation of phosphorylation was determined and plotted in an amplitude-based hierarchical fashion. Background phosphorylation of the array was determined from the exponent describing the amplitude behavior of the 500 least phosphorylated peptides (which are assumed not to contain phosphorylation derived from a relevant biological signal). Peptides of which the average phosphorylation minus 1.96 times the standard deviation was higher than background were considered to represent true phosphorylation events (P<0.05). Peptide sets that showed statistically significant changes compared to the background were subsequently subjected to elective Markov analysis and the resulting binary values were also analyzed in cohorts of peptides representing a read out for the same signal transduction element, collapsing results towards human interpretable format. Significance analysis was performed using a minimal modification for the algorithm originally developed for microarray analysis (www-stat.stanford.edu/~tibs/SAM/) and was essentially done as described earlier\(^15\).

PROTEIN EXTRACTION AND CONCENTRATION DETERMINATION

Cells were harvested in lysis buffer (25 mmol/l HEPES, 2 mmol/l EDTA, 150 mmol/l KAc, 0.1% NP40, 10 mmol/l NaF, 1 mmol/l PMSF, 1 mmol/l DTT, aprotinin, leupeptide and prostatine (each 1 µg/ml) pH 7.2) followed by four cycles of freezing (liquid nitrogen) and thawing (37°C). Lysates were centrifuged (10 min, 12,500 xg, RT) and supernatant was stored at -80°C until use. Protein concentrations were determined using the BioRad DC protein assay (BioRad, CA, USA). Absorbance was measured at 750 nm on an EL800 universal microplate reader (Bio-Tek instruments, VT, USA).

WESTERN BLOTTING

Total protein extracts (20 µg) from control and treated cells were separated on 10% sodium dodecyl sulphate polyacrylamide (SDS-PAGE) gels and transferred to nitrocellulose membrane (GE Healthcare Europe, Diegem, Belgium). After blocking with 5% skim milk, membranes were probed with primary antibodies (Supplementary table S1). The target proteins were visualized using an enhanced chemiluminescence SuperSignal West Dura detection system (#34076; Pierce, IL, USA). GAPDH (#CB1001; Calbiochem, CA, USA) was used as loading control.
QUANTIFICATION OF APOPTOTIC AND NECROTIC CELL DEATH

Caspase-3 activity in 20 µg total cellular protein was measured as described previously\textsuperscript{16} using an FL600 microplate fluorescence reader (Bio-Tek instruments) with excitation and emission set at 380 and 460 nm, respectively. To detect necrotic cell death (leaky plasma membrane), cells were incubated for 15 min with sytox green nucleic acid stain (Molecular Probes, OR, USA), as previously described\textsuperscript{13}.

METABOLIC ACTIVITY

Metabolic activity of cells was determined using WST-1 reagent following the instructions of the manufacturer (Roche, CA, USA). Cells exposed to 5 mmol/l H\textsubscript{2}O\textsubscript{2} were used as a control for complete suppression of metabolic activity.

INHIBITION OF PHOSPHORYLATION

To determine the contribution of the survival/proliferation PI3K/Akt and/or autophagy/AMPK pathways to CSE-mediated protection of DLD-1 and Jurkat cells to apoptosis, we used the PI3K inhibitor LY294002 (50 µM for DLD-1, 25 µM for Jurkat), the ERK1/2 inhibitor U01260 (10 µM) and the AMPK inhibitor 5-iodotubercidin (0.1 µM) in the absence or presence of CSE and/or anti-Fas. Cells were pre-treated with the inhibitors for 30 min.

STATISTICAL ANALYSIS

Data are expressed as the means ± SD of at least 3 independent experiments that were each performed in duplicate. One way analysis of variance (ANOVA) in combination with a Bonferroni correction was used to determine the statistical significance of the differences between groups. A P value of <0.05 was considered statistically significant.

RESULTS

DISSECTION OF THE MOLECULAR PATHWAYS MODULATED BY CIGARETTE SMOKE

First, we determined the effect of various concentrations cigarette smoke-saturated medium (e.g. cigarette smoke extract; CSE) on cell viability and the metabolic activity of DLD-1 cells (model for human intestinal epithelial cells) and Jurkat cells (model for human T-lymphocytes) (Figure 1). Concentrations up to 20% CSE did not cause significant levels of necrosis in either cell type (Figure 1A). In fact, these conditions significantly enhanced the metabolic activity of both cell types compared to untreated control cells (Figure 1B and C, for DLD-1 and Jurkat, respectively). High
concentrations of 50 and 100% CSE strongly reduced the metabolic activity of DLD-1 cells and Jurkat cells and causes massive necrosis.

**DISSECTION OF THE CELL SIGNALING PATHWAYS MODULATED BY CIGARETTE SMOKE**

To identify possible molecular signaling pathways that are modulated by CSE, we resorted to kinome profiling, an unbiased approach allowing the generation of comprehensive description of cellular signaling without the need of a priori assumptions as to the pathways affected by smoke. DLD-1 and Jurkat cells incubated with or without 20% CSE for 30 min were used for this analysis. The data were subjected to Markov analysis and the results reveal the complement of kinase changes upon CSE challenge. For 20% CSE 141 DLD-1 and 184 Jurkat phosphorylated substrates
were detected (Supplementary Table S2A and B). A schematic representation of all differentially phosphorylated peptides can be found in Supplementary Figure S1. Overall, analyses revealed that CSE activates signals associated with inhibition of ligand-induced apoptosis (Figure 2), while cell proliferation pathways and autophagy were induced (Supplementary Figure S1). Individual proteins were analyzed by Western blotting and in most cases confirmed the kinome analysis (Figure 3A and B, for DLD-1 and Jurkat cells, respectively), with some exceptions described below. Exposure of DLD-1 cells to 20% CSE increased the phosphorylation of STAT5, ERK1/2, Bad and protein levels of Bcl-XL (anti-apoptotic/pro-survival); increased phosphorylation of Akt, GSK3β and protein levels of cyclin D1 (pro-proliferation); and increased phosphorylation of AMPK and lipidation of LC3 (anti-apoptotic/pro-

Figure 2. Provisional signal transduction schemes - impression of kinome profiling results. Paired cultures of DLD-1 cells and Jurkat cells were challenged with vehicle or 20% CSE followed analysis for kinase activity using peptide arrays. Results were collapsed on selective signal transduction categories and expressed in gray scale (representing the number of peptides phosphorylated as a fraction of all peptides in that category). Vehicle and CSE effects were contrasted. The results provide an overview of anti-apoptosis and pro-survival pathways active in DLD-1 cells and Jurkat cells and the effects of CSE thereon.
Figure 3. Cell signaling pathways activated by CSE. DLD-1 and Jurkat cells were exposed for 30 min to 0% and 20% CSE and expression of phosphorylated forms of Src, STAT5, ERK1/2, Bad, Akt, GSK3β and AMPK, and protein expression of Bcl-XL, cyclin D1 and LC3 in DLD-1 (A) and Jurkat (B) cells was analyzed by Western blotting. GAPDH was used as loading control.

autophagy) (Figure 3A). The CSE-induced phosphorylation of AMPK in DLD-1 was not detected in the kinome analysis, possibly by low basal levels in control cells and/or the fact that only 1 AMPK target peptide was phosphorylated in the kinome array in those cells. In Jurkat cells, 20% CSE decreased phosphorylation of ERK1/2, but increased protein levels of Bcl-XL (anti-apoptotic/pro-survival); increased phosphorylation of Akt, GSK3β and protein levels of cyclin D1 (pro-proliferation); and increased phosphorylation of AMPK (anti-apoptotic/pro-autophagy) (Figure 3B). Together, these data provide novel data on the effects of cigarette smoke in two different in vitro models with relevance for IBD and demonstrate that the main action of cigarette smoke is activation of anti-apoptotic and pro-survival pathways.

SMOKE EXERTS CYTOPROTECTIVE EFFECTS UPON PRO-APOPTOTIC STIMULATION

Next, we analyzed whether the CSE-induced anti-apoptotic/pro-survival pathways translate into functional consequences with respect to protection against pro-apoptotic triggers, including anti-Fas, CM and menadione. Anti-Fas-induced caspase-3 activity was dose-dependently inhibited by CSE (10-20%) in both DLD-
1 (Figure 4A) and Jurkat cells (Figure 4B). Similarly, CM- and menadione-induced caspase-3 activity was dose-dependently inhibited by CSE (5-20%) in DLD-1 cells (Figure 4A). Jurkat cells were not sensitive for CM- and menadione-induced apoptosis. In line with the reduction in caspase-3 activity, CSE also reduced the level of PARP cleavage (marker for late apoptosis) induced by anti-Fas, CM or menadione in DLD-1 and Jurkat cells (only for anti-Fas for the latter) (Figure 4C and D). The CSE inhibitory effects were most pronounced for menadione-induced apoptosis. CSE alone did not induce caspase-3 activity. Similar results were obtained when these experiments were performed with other intestinal (HCT116, SW480, Caco-2) or lymphocyte (MOLT-4) cell lines (Supplementary Figure S2 A-D).

**Figure 4.** CSE inhibits death receptor- and oxidative stress-induced apoptosis in DLD-1 and Jurkats cells. (A) Caspase-3 activity in total extracts of DLD-1 cells exposed to anti-Fas, CM or menadione in combination with increasing concentrations CSE. (B) Caspase-3 activity in total extracts of Jurkat cells exposed to anti-Fas in combination with increasing concentrations CSE. Maximal caspase-3 activity is presented as 100%. Data represent means ± SD of at least three independent experiments. *P<0.05 vs. control. (C,D) Western blot analysis of PARP showing that CSE inhibits anti-Fas-induced cleavage to the 89 kDa form in DLD-1 (C) and Jurkat (D) cells. GAPDH was used as loading control.

**DISSECTION OF THE RELATIVE IMPORTANCE OF CSE-INDUCED SIGNALING PATHWAYS IN CYTOPROTECTION**

Several pro-apoptotic pathways are simultaneously suppressed by CSE. In order to determine which pathways contribute to the anti-apoptotic effect of CSE, we blocked the AMPK pathway (by 5’-iodotubercidin), the PI3K survival pathway (by LY294002)
or the ERK1/2 pathway (by U01260) in anti-Fas/CSE co-treated DLD-1 and Jurkat cells (Figure 5). The CSE-induced reduction in caspase-3 activity in anti-Fas-treated Jurkat cells was significantly reversed by the AMPK-inhibitor (Figure 5A). A similar trend was observed for DLD-1 cells, but was not statistically significant. Inhibition of the PI3K and ERK1/2 pathway significantly increased the caspase-3 activity in anti-Fas/CSE co-treated cells (Figure 5B and C), however, this was also observed in anti-Fas treated cells. This implies that the PI3K or ERK1/2 pathways are already activated by anti-Fas and a potential additive effect of CSE cannot with certainty be deduced from these inhibitor experiments (Figure 5B and C). Taken together, these data show that the anti-apoptotic effect of CSE is mediated through multiple pathways and that inhibition of the AMPK pathway may provide a degree of specificity between the epithelial compartment and T-cell compartment respectively. The putative

Figure 5. The anti-apoptotic effect of CSE is not solely dependent of the AMPK, PI3K or ERK1/2 pathway. DLD-1 and Jurkat cells were exposed to anti-Fas and CSE with or without (A) the AMPK inhibitor 5'-iodotubercidin, (B) the PI3-kinase inhibitor LY294002 (C) the ERK1/2 inhibitor U01260. Caspase-3 activity was presented as fold-change compared to control; control values were set at one. Data represent means ± SD of at least three independent experiments. *P<0.05 vs. anti-Fas treated cells, #P<0.05 vs. cells co-treated with anti-Fas and inhibitor, $P<0.05 vs. anti-Fas/CSE co-treated cells.
Figure 6. Nicotine, CO and acrolein contribute to the anti-apoptotic effects of cigarette smoke. Caspase-3 activity in DLD-1 cells after co-treatment with anti-Fas, CM or menadione and increasing concentrations of (A) the water-soluble fraction of CSE, (B) Nicotine (0-10 µM), (C) 100% CO-saturated medium, (D) CORM-2 or (E) acrolein (0-100 µM). Data represent means ± SD of at least three independent experiments. *P<0.05 vs. control, #P<0.05 vs. CORM-treated control bars.

involvement of the ERK1/2 or the PI3K pathway in the CSE-mediated anti-apoptotic effect cannot be established, since those pathways are already activated by anti-Fas.

EFFECTS OF SMOKE CONSTITUENTS

Finally, we aimed to determine which compounds in cigarette smoke contribute to the anti-apoptotic effect of CSE. In a setup that can be compared to a “water pipe”, we first passed the smoke of 2 research cigarettes through 25 ml of water, after which it was passed through 25 ml medium. The anti-apoptotic effect completely shifted to the “water-trap” indicating that the protective compound(s) were efficiently and
completely solubilized in water (Figure 6A). Cigarette smoke compounds that are highly water-soluble are nicotine and acrolein, but may also include gases like CO. These compounds were further evaluated for their anti-apoptotic properties on anti-Fas-treated DLD-1 cells. Nicotine decreased anti-Fas induced caspase-3 activity with 31 ± 9.9% (10 µM; Figure 6B). Incubation with CO-saturated medium yielded a reduction of 46 ± 2.5% (Figure 6C). CM- and menadione-induced apoptosis were also inhibited by CO-saturated medium. CORM2 and acrolein dose-dependently decreased caspase-3 activity up to 59 ± 1.9% (200 µM; Figure 6D) and 69 ± 3.5% (100 µM; Figure 6E), respectively. Tested concentrations had no effect on the metabolic activity of DLD-1 cells, nor did it induced necrosis (data not shown). We conclude that cigarette smoke contains multiple water-soluble components exhibiting a cytoprotective action on intestinal epithelial cells and T-lymphocytes, possibly explaining the multitude of cytoprotective pathways induced.

DISCUSSION

In this study, we show that cigarette smoke induces phosphorylation of kinases that are involved in anti-apoptotic (STAT5B/ERK1/2) and pro-survival pathways (AMPK/Akt/PI3K) in intestinal epithelial cells and T-lymphocytes. This was accompanied by enhanced levels of anti-apoptotic Bcl-XL/Bcl-2 proteins, pro-survival proteins GSK3β, cyclin D1 and lipidation of the autophagy marker LC3. Cigarette smoke enhanced the metabolic activity of intestinal epithelial cells and T-lymphocytes and suppressed anti-Fas-, cytokine- and oxidative stress-induced apoptosis. The smoke components nicotine, CO and acrolein all suppressed apoptosis to variable degrees and inhibition of single pro-survival kinase pathways did not block the protective effect of cigarette smoke. Our data show that cigarette smoke induces a plethora of pro-survival pathways and protects intestinal epithelial cells and T-lymphocytes against inflammation-induced apoptosis. Cigarette smoke may aggravate CD by enhancing the resistance of T-lymphocytes to apoptosis, while it may ameliorate UC by preventing excessive damage to the intestinal epithelial layer (Figure 7).

The kinome profiling combined with the Western blot analysis of protein phosphorylation revealed a smoke-dependent stimulation of pro-survival pathways (AMPK/Akt/PI3K and GSK3β/cyclin D1/LC3) and anti-apoptotic pathways (STAT5B/ERK1/2 and Bcl-XL/Bcl-2). These observations correspond well to previous studies, where effects of cigarette smoke were investigated in COPD and lung cancer using micro array analysis and/or proteomic approaches. Similar kinase pathways were found to be affected by smoke, supporting a common role by which cigarette smoke
Figure 7. Proposed model for the dichotomal effects of cigarette smoke on Crohn's disease and ulcerative colitis explained by inhibition of apoptosis. Upper panel: Normal colon with mild apoptosis activity and mixed infiltrate restricted to the top of villi. Crypt architecture is preserved. The crypts are parallel to each other and extend from the luminal surface to the muscularis mucosae. Upper left panel: Ulcerative colitis with high apoptosis rates in the epithelium. Severe diffuse crypt atrophy and diffuse chronic inflammation with mixed infiltrate. Crypt distortion includes crypt branching and loss of parallelism. Lower left panel: Crohn's disease with diffuse transmucosal chronic inflammation including lamina propria infiltrate, mainly T-lymphocytes. Abnormal crypt architecture. Upper right panel: Ulcerative colitis after smoking with less apoptosis activity in the epithelium. Restoration of crypt architecture and less mucosal infiltrate. Lower right panel: Crohn's disease after smoking with severe transmucosal chronic inflammation and T-lymphocyte infiltrate. Less apoptosis of T-lymphocytes shifting toward excessive proliferation.
acts throughout the human body. Cigarette smoke seems to act, at least partly, through the AMPK pathway as AMPK inhibition reversed the CSE-cytoprotective effect in Jurkat cells and a similar trend was observed for DLD-1 cells. A potential role of the PI3K and/or ERK pathway in the anti-apoptotic effect of smoke under inflammatory conditions remains obscure, as these are already activated by anti-Fas. However, key factors in these pathways are activated by CSE in the absence of anti-Fas and therefore may provide a basal protection to apoptosis.

Maintenance of the colonic epithelium is the result of a delicate balance between cell proliferation at the base and cell death (apoptosis) at the surface epithelium. Apoptosis must therefore be highly regulated and minor perpetuations in epithelial apoptosis rates may disturb the epithelial barrier and allow luminal pathogens to pass the epithelium. The ongoing exposure of pathogens triggers the immune system and causes inflammation of the colon and further increases epithelial cell damage. UC is characterized by high epithelial cell apoptosis rates\(^{19}\) and impaired barrier function that lead to increased intestinal permeability. In fact, an increased intestinal permeability to harmful substances may be one of the initiating factors in the development of UC. Several studies report that smoking may act to tighten the gut and confer its beneficial effect upon UC by reducing intestinal permeability\(^ {20,21}\).

Here we show that smoking inhibits apoptosis in colonic epithelial cells and increases the metabolic state of these cells, thereby improving epithelial survival and barrier function.

Apoptosis also controls the life span of invading immune cells during inflammation and thereby modulates inflammatory responses via limiting expansion of activated T-cells. This process is of key importance in the intestinal mucosa, as it limits the size of activated T-cell compartment and thereby prevents uncontrolled expansion of the inflammatory response leading to tissue damage. The resistance of mucosal T-cells to undergo apoptosis is strongly implicated in the pathogenesis of IBD. T-cells from CD patients have an intrinsic defect in activation-dependent apoptosis, which leads to an inappropriate cellular expansion and an ongoing inflammatory reaction\(^ {22}\). This defect in apoptosis has been attributed to an imbalance between two mitochondrial proteins, the anti-apoptotic Bcl-2/Bcl-XL and the pro-apoptotic Bax\(^ {23,24}\). Here, we show that exposure to cigarette smoke enhances the levels of Bcl-2/Bcl-XL and thereby protects T-cells against inflammation-induced apoptosis. This indicates that the detrimental effect of smoking on CD pathogenesis could be due to a further enhanced resistance of T-cells against apoptosis. Smoke exposure has been shown to increase T-cell populations. Tanigawa et al. reported that cigarette
smokers have enhanced numbers of circulating T-lymphocytes\textsuperscript{25}. Also, Verschuere \textit{et al.} reported a significant increase and recruitment of various immune cell types into murine Peyer’s patches such as CD4+ T-cells, CD8+ T-cells and regulatory T-cells after chronic smoke exposure, although this was not associated with tissue damage\textsuperscript{26}. The induction of apoptosis in T-cells is thought to be a key therapeutic action of several immunosuppressive drugs and anti-TNF agents are used in the treatment of CD\textsuperscript{27-29}. Therefore smoking might impair the effect of these drugs, however, clinical studies are not conclusive in this respect.

In rheumatoid arthritis (RA), another autoimmune disease characterized by resistance to apoptosis and smoke-related disease development\textsuperscript{30,31}, a specific genetic variant of \textit{PTPN22} was found to be associated with RA and heavy smoking\textsuperscript{32,33}. As \textit{PTPN22} is involved in T-cell receptor signaling, it further supports the importance of T-cell signaling in autoimmune diseases like IBD. While this cigarette smoke-induced inhibition of apoptosis is aggravating CD, it is protective against UC. Although smoking ameliorates UC, we have to stress that smoking leads to many health problems that results in oxidative stress-induced cell damage. For example, smoking is known to be an independent risk factor for carcinogenesis in Barrett’s esophagus and colorectal neoplasia\textsuperscript{34-36}. As a correlation exists between inflammation and the risk of gastrointestinal cancer\textsuperscript{37,38}, one could speculate about the role of cigarette smoking in inflammation-associated carcinogenesis in the colon. However, no data is available about an increased risk of colon cancer neither in smoking CD patients with colonic disease nor in smoking UC patients who are in remission upon smoking.

In order to pinpoint potential active mediators responsible for the effects smoking exerts, we analyzed the anti-apoptotic effect of purified and water-soluble components from cigarette smoke in T-lymphocytes and intestinal epithelial cells. We observed significant cytoprotection for all components tested, suggesting the involvement of various cytoprotective pathways. Nicotine is the most well studied compound of cigarette smoke and may be beneficial by increasing mucin synthesis\textsuperscript{19}, gut permeability\textsuperscript{20} and by decreasing pro-inflammatory cytokines\textsuperscript{40} mainly through its action on the nicotinic acetylcholine receptor on immune cells\textsuperscript{41}. In this report, we show a significant cytoprotective action of nicotine at concentrations similar to concentrations found in blood of smokers\textsuperscript{42}. However, conflicting data of therapeutic effects of nicotine exists and other compounds may contribute more to the cytoprotective action of smoke. Recent studies on acrolein are in line with our results as it was shown to exert anti-inflammatory actions through multiple signal transduction pathways, including mitogen-activated protein kinase (MAPK)
pathways\textsuperscript{43}, inhibition of NF-κB and by activating Nrf2, thereby inducing anti-inflammatory genes such as HO-1\textsuperscript{44}. Although several studies indicate that acrolein induces apoptosis and necrosis in various cell types\textsuperscript{45}, acrolein was also found to inhibit apoptosis by influencing redox-sensitive caspase signaling cascades\textsuperscript{46}, which is consistent with our findings. Studies on CO/CORMs exposure seem to be more consistent in proving a potent anti-inflammatory action, as it was shown to protect cells through anti-inflammatory, anti-proliferative and anti-apoptotic signaling in several models of colitis\textsuperscript{47,48}. These protective effects of CO involve inhibition of T-cell activation and proliferation, suppressing inflammation supposedly by downregulating of pro-inflammatory cytokines and modulation of numerous cellular targets including MAPKs. Depending on stimulus and timing, their activity is differentially regulated by CO\textsuperscript{49,50}. Here, we show a cytoprotective action of CO and CORMs against inflammation-induced apoptosis, which is consistent with other studies on CO/CORMs\textsuperscript{51}.

Cigarette smoke constituents exert both immunosuppressive and immunogenic effects and we show a general anti-apoptotic effect that could explain the dichotomy of cigarette smoke on CD and UC. It is important to emphasize that components in cigarette smoke do not suppress or enhance inflammation by a single specific mechanism, but by a combination of protein/kinase pathways that collectively result in anti- (UC) or pro- (CD) inflammatory responses.

Further studies on smoke components are needed in order to provide novel strategies to maximize the beneficial effects of smoking in UC and minimize the deleterious effects on CD and general health.

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Supplementary Table S1. Antibodies used in this study.

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<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
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Supplementary Table S2A and S2B.

On request (anoukregeling@gmail.com).
Supplementary Figure S1. Provisional signal transduction schemes - impression of kinome profiling results. Paired cultures of DLD-1 cells and Jurkat cells were challenged with vehicle or 20% CSE followed by analysis for kinase activity using peptide arrays. Results were collapsed on effective signal transduction categories and expressed in gray scale (representing the number of peptides phosphorylated as a fraction of all peptides in that category). Vehicle and CSE effects were contrasted. The results provide an overview of the signaling pathways active in DLD-1 cells and Jurkat cells and the effects of CSE thereon.
Supplementary Figure S2. Caspase-3 activity in total extracts of (A) HTC116 cells, (B) Caco-2 cells, (C) SW480 cells and (D) MOLT-4 cells exposed to anti-Fas, CM or menadione in combination with increasing concentrations CSE. Maximal caspase-3 activity is presented as 100%. Data represent means ± SD of at least three independent experiments. *P<0.05 vs. control.