Chapter 10

Animal Inhalation Models to Investigate Modulation of Inflammatory Bowel Diseases

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Additional information is available at the end of the chapter

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Abstract

Inflammatory bowel diseases (IBDs) comprise primarily two disease manifestations, ulcerative colitis (UC) and Crohn's disease (CD), each with distinctive clinical and pathological features. Environmental and clinical factors strongly affect the development and clinical outcomes of IBDs. Among environmental factors, cigarette smoke (CS) is considered the most important risk factor for CD, while it attenuates the disease course of UC. Various animal models have been used to assess the impact of CS on intestinal pathophysiology. This chapter examines the suitability of animal inhalation/smoke exposure models for assessing the contrary effects of CS on UC and CD. It presents an updated literature review of IBD mouse models and a description of possible mechanisms relevant to relationships between IBD and smoking. In addition, it summarises various technical inhalation approaches, in the context of mouse disease models of IBD.

Keywords: inhalation, inflammatory bowel disease, animal models, cigarette smoke, ulcerative colitis, Crohn's disease

1. Introduction

Inflammatory bowel disease (IBD) is a chronic inflammatory condition of the gastrointestinal tract encompassing two main disease manifestations, Crohn's disease (CD) and ulcerative colitis (UC) [1].

CD and UC have many similarities in symptoms and disease phenotypes, making diagnosis challenging [2]. Currently, criteria for distinguishing these two manifestations are based exclusively on histopathological and endoscopic examinations [3]. Thus, UC is defined as a chronic, non-transmural inflammatory disease characterised by diffuse mucosal inflammation

involving only the colon. Its primary clinical symptom is bloody diarrhoea [2, 4–7]. As UC is an inflammatory disease, the state of the immune system is a fundamental aspect of the disorder, with an atypical T helper cell (Th)2 response, mediated by natural killer T cells that secrete interleukin (IL)-13 [1, 8, 9]. CD is a relapsing, transmural inflammatory disease that may affect the entire gastrointestinal tract. Its major clinical symptom is abdominal pain or nonspecific abdominal symptoms and bloody diarrhoea is rare. The T cell profile in CD is different from that of UC and, in fact, a Th1 cytokine profile is dominant in patients with CD [4, 7, 10, 11]. Notably, innate immune responses are similarly activated in both CD and UC [12]. Several studies suggested that IBD pathologies result from an inappropriate inflammatory response to intestinal microbes in a genetically susceptible host, with consequent alteration of the intestinal epithelium.

During IBD development, the paracellular space in the intestinal epithelium becomes more permeable, impacting defensive strategies naturally activated by specialized epithelial cells, including goblet and Paneth cells [13–16]. This process primes a positive feedback loop, with increased exposure to the intestinal microbiota, leading to amplification of the inflammatory response. Observations in patients or animal models show that host-microbiome interactions and microbiome fluctuations play prominent roles in such inflammatory processes [17, 18]. However, whether these alterations contribute to the disease, or simply reflect secondary changes caused by the inflammation, is still under debate.

Indeed, the basic aetiology of IBD is still unclear and the potential factors contributing to the pathogenesis of the disease, such as dysbiosis, epithelial and/or immune system dysfunctions and oxidative stress, represent the major research topics in the IBD field. Moreover, new area of interest arose from the necessity of understanding the potential environmental causes behind the disease onset.

Among the environmental factors associated with IBDs, the most significant causes are cigarette smoke (CS) and nicotine, and these inversely affect the risk and course of UC and CD. The relationship between smoking and IBD has been known for many years, with the first report of a negative correlation between IBD and smoking, in a cohort of UC patients, published 40 years ago [19]. Since then, there have been numerous epidemiological, clinical and pre-clinical studies describing the dual effects of active smoking in the two forms of IBD [20, 21]. CS is associated with a higher risk for developing CD and a worse outcome in CD patients. In contrast, UC is considered a non-smokers' disease, with a significantly lower risk of disease development in current smokers. Despite the considerable research on smoking and IBD, the molecular mechanisms for CS-induced impacts on IBD development, as well as the specific CS components responsible, are not well understood [22].

To better understand the different aetiological factors in the onset of IBD, a variety of disease models were developed. Human and *in vitro* studies have historical limitations because of design complexity, duration and cost or, for *in vitro* studies, the lack of translational applicability. Therefore, animal models are advantageous by allowing *in vivo* experiments to be conducted under more easily controlled conditions than those in human studies, while providing the organism complexity lacking in *in vitro* systems. Increased knowledge of mucosal immunity and host-microbiome interactions and dynamic, as well as the availability of new

genetic engineering technologies, enabled the development of numerous murine models that, in turn, substantially increased the understanding of intestinal inflammatory processes [23, 24]. Arguably, none of these models can completely recapitulate the complexity of human IBD, but they can provide valuable information about major aspects of the disease, thereby enabling a common set of principles of human IBD pathogenesis to be established.

This book chapter reviews key studies conducted in animal inhalation/smoke exposure models aimed at evaluating the different modulation of UC and CD by CS. The application of inhalation technology to rodents, reproducing the clinical effects of smoking on colonic inflammation, will increase the chances of identifying new anti-inflammatory molecular mechanisms and possibly therapeutics, finally increasing the chances of IBDs defeat.

2. Technical aspects of inhalation

2.1. Methods of acute and chronic pulmonary delivery of aerosols to rodents

The technical means for pulmonary delivery of aerosols (either small molecules, proteins or mixtures) may employ either direct intratracheal administration or, alternatively, inhalation exposure, the latter often requiring restraint of animals.

For acute pulmonary delivery of an agent, intratracheal administration may be ideal. Its main advantages are that it requires little infrastructure or equipment and can be performed in a basic *in vivo* lab environment [25]. In addition, dose delivery can be accurately and reproducibly estimated [26]. However, this method also has several shortcomings, such as need for anaesthesia, inability to administer volatile agents or gases and unequal distribution in the lungs, resulting in minimal exposure to the alveoli. Overall, such concerns make intratracheal administration a less suitable method for subchronic or chronic pulmonary delivery.

For subchronic or chronic administration of aerosols to rodents, repeated inhalation exposure systems are preferred. Thus, animals are exposed to aerosols within a confined environment for a fixed daily duration. In the field of toxicology, testing guidelines for repeated dose exposure for toxicological assessments, such as the OECD TG413 guideline, recommend up to 6 h per day exposure for a 90 day exposure period. However, for therapeutic or disease modelling purposes, the exposure period must be determined empirically, based on the effective dose and the time needed for the target biological effect to occur. Importantly, exposure systems must enable consistent delivery of aerosols, at concentrations that are stable during the exposure period, and with appropriate aerosol properties to enable efficient inhalation and uptake [27].

Principally, two types of exposure chambers are routinely used to administer aerosols to rodents, whole body or nose-only exposure chambers, each with its own advantages and disadvantages [27]. Whole body exposure systems are restraint free, as the animals are placed into an exposure chamber, either in a cage or on a mesh or grid surface, depending on the specific system. Both chambers are technically simple, assuming sufficient infrastructure (aerosol

generation and functional chambers). Both also enable exposure of large numbers of animals, for example, chambers of >700 L may each accommodate approximately 200 mice. The freedom of movement of animals during exposure results in minimal stress, although the animals require training to adjust to grid-caging systems and food is typically withdrawn to minimise oral uptake of aerosol constituents. One criticism of whole body exposures is that there is a high potential for compound uptake through non-inhalation routes because animals have surface contact with aerosol deposits on the cage surfaces and on their fur. In historical studies, up to 60% of aerosol constituents on the fur (pelt burden) were ingested following whole body exposures [28] and transdermal uptake may also be significant for some compounds. Because the skin is an effective barrier for drug transport, only potent drugs with appropriate physicochemical properties (low molecular weight and adequate solubility in aqueous and non-aqueous solvents) are suitable candidates for transdermal delivery [29–31]. Such mixed uptake mechanisms potentially occurring in whole body exposure systems complicate both dose estimations and require deconvolution of uptake amounts through oral/transdermal and inhaled routes.

Nose-only exposure chambers require restraint of the animals to permit only the head (nose) to be exposed to the test aerosol. This has the major advantage of decreasing deposition of aerosol constituents on the pelts, resulting in less oral uptake from grooming behaviour [32]. However, there are also disadvantages with this system, including technical asphyxiation (animal movements in the exposure tube may cut off their air supply); therefore, constant monitoring during the exposure period is required. In addition, because of stress associated with restraint in nose-only exposure systems, training is required to adapt animals to the technical procedures. Vehicle or fresh air exposures are also needed to help distinguish such stress-related effects from treatment effects [33]. The daily execution of nose-only exposures requires that animals be individually inserted into the exposure tubes, a technical aspect that may limit the numbers of animals that can be used in the experiments.

2.2. Dose translatability

Measurement of dosages in an *in vivo* inhalation experiment is dependent upon many parameters, including deposition of the agent to the lungs (which itself is dependent upon aerosol droplet size), respiratory minute volume and body weight of the animal. This relationship is generally described by the following formula [34]:

$$DD = \frac{C \times RMV \times D \times IF}{\text{Body weight (kg)}} \quad (1)$$

where DD is the delivered dose (mg/kg); C is the concentration of substance (mg/L); RMV is the respiratory minute volume (L/min) and IF is the inhalable fraction.

Among these parameters, the respiratory minute volume is important to determine the availability of compound for deposition and exchange in the lungs. This parameter may be calculated using allometric formulae relating body weights to minute volumes in laboratory animals [35, 36]. The alternative, direct measurement of the minute volume, as can be

performed when nose-only exposure tubes are used (head-out plethysmography measurements), is preferable as it would enable the researcher to control any effects of test item on the minute volume, when calculating the estimated dosage.

Important for *in vivo* disease modelling is the translation of the animal models to human therapeutics or treatment regimen. This will require an estimation of human equivalent dose (HED), based on the animal data. The most commonly used method to convert to HED is with a body surface area conversion factor [37]. Alternatively, a mg/kg conversion factor may be applied, though this typically will result in a lower safety margin and higher HED values, compared with the body surface area conversion. HED is generally described by the following formula [37]:

$$\text{HED} = \frac{\text{animal dose (mg/kg)} \times \text{animal } K_m}{\text{human } K_m} \quad (2)$$

where K_m is the correction factor reflecting the relationship between body weight and body surface area (e.g. human $K_m = 37$; mouse $K_m = 3$; rat $K_m = 6$ and dog $K_m = 20$).

3. Overview of animal IBD models

The various types of animal models developed to study IBD may be divided into several categories depending on: the method of inducing the pathology (*chemically induced, bacteria-induced or genetically engineered*); the IBD subtype modelled in the animal (*UC or CD*); the site of inflammation (*colon, ileum, both sites or systemic*); and, in genetically engineered models, the gene modification strategy (*conventional transgenic* (*Tg*) *or knockout* (*KO*), *cell-specific conditional Tg or KO, inducible KO, knock-in, innate, mutagen-induced or spontaneous models*) [23, 38, 39]. The total number of IBD mouse models is growing, especially because of current genetic engineering approaches that accelerate development of new strains, so far, over 74 genetically engineered mouse models were reported to spontaneously develop intestinal inflammation [38]. The full description of all IBD models is beyond the scope of this chapter. However, **Table 1** summarises the most significant IBD murine models, highlighting their methods of pathology induction, IBD subtypes, sites of inflammation and mechanism of action (**Figure 1**). More detailed reviews of the different mouse models of IBD are available (e.g. see Refs. [23, 40, 41]).

There is a close agreement in many pathological findings among experimental IBD models and human disease. These include the molecular pathways and histological features of tissue injury, dysfunction of the immune system (including impact of the microbiome), genetic heterogeneity and primary defects in mucosal barrier function. All pathologies have been well established in several experimental models of colitis; therefore, these models closely resemble aspects of the human diseases. These common features enable exploration of specific pathological mechanisms, facilitating development of new therapeutic approaches. However, none of these models fully reflects human IBD, with each representing rather a small tile of a mosaic. This hinders a generalised view of the systemic consequences of IBD, often masking possible extra-intestinal implications [42].

IBD model	Model category	IBD subtype	Site of inflammation	Mechanism	References
DSS	Chemically induced	UC	Colon	Epithelial cell damage	[147, 148]
TNBS		**CD**/UC	Colon	Hapten-dependent immunogenic response	[149]
DNBS		CD/UC	Colon	Hapten-dependent immunogenic response	[150]
Oxazolone		UC	Colon	Hapten-dependent immunogenic response	[151]
Acetic acid		UC	Colon	Epithelial cell damage	[152]
Carrageenan		UC	Colon	Epithelial cell damage	[153]
Indomethacin		CD	**Small intestine** Colon	Epithelial cell damage	[154]
Iodoacetamide		UC	Colon	Sulphydryl (SH) compound (e.g. glutathione) blocker	[155]
DNCB		**UC**/CD	Colon	Hapten-dependent immunogenic response	[156]
Salmonella induced	Bacterially induced	UC	Colon	Bacterial colonisation-induced inflammation	[157]
Adherent invasive *E. coli*		UC	**Colon** Small intestine	Bacterial-dependent epithelial cell damage	[158]
C3H/HejBir	Spontaneous	CD	**Small intestine** Colon	Epithelial cell dysfunction	[39]
SAMP1/4it		CD	Small intestine	Epithelial cell dysfunction	[40]
IL-10$^{-/-}$	Genetically engineered/ knockouts (KO)	CD	**Small intestine** Colon	Impaired Treg function	[74]
TGF-β$^{-/-}$		UC/CD	Systemic	Macrophage hyperactivation and impaired Treg function	[159]
IL-2$^{-/-}$		UC	Colon/systemic (no small intestine)	Impaired T cell/Treg function	[160]
NOD2$^{-/-}$		CD	Small intestine Colon	NF-κB and TLR2 signalling dysregulation	[161]
A20$^{-/-}$		UC/CD	Colon Small intestine	TNF-induced NF-κB signalling dysregulation	[162]
MDR1A$^{-/-}$		UC	Colon	Accumulation of bacterial products and increased T cell activation	[163]
Gαi2$^{-/-}$		UC	Colon	Impaired T/B cell function and epithelial cell damage	[164]
TCRα$^{-/-}$		UC	Colon	Th2-type inflammation	[75]
IL-23$^{-/-}$		CD	Small intestine Colon	Impaired Th17 cell function	[165, 166]

IBD model	Model category	IBD subtype	Site of inflammation	Mechanism	References
XBP1$^{-/-}$	Genetically engineered, conditional KO	CD	**Small intestine** Colon	Loss of Paneth and goblet cells with impairment of mucosal defence	[167]
NEMO$^{-/-}$		CD	Small intestine/ colon	NF-κB signalling dysregulation	[168]
IL-7 Tg mice (IL-7 overexpression)	Transgenic mouse	UC	Colon	CD4+ T cell infiltration-dependent inflammation	[169]
STAT4 Tg mice (STAT4 overexpression)		CD	Small intestine Colon	Th1-type inflammation	[170]
HLA-B27 Tg mice		UC/CD	Small intestine Colon	Bacterial sensitisation	[171]
DNN-cadherin/ keratin8$^{-/-}$		CD	Colon	Epithelial cell dysfunction	[172]
TNF$^{\Delta ARE}$	Mutation knock-in	CD	Small intestine	TNF-α overproduction	[64]
CD45RB high-transfer	Adoptive transfer	CD	**Small intestine** Colon	IL-12-driven Th1 hyper-response	[173]

Table 1. Classification of animal models of IBD. IBD subtype and site of inflammation predominantly addressed by the model, where applicable, are shown in bold font. DSS, dextran sulfate sodium; IBD, inflammatory bowel disease; DNBS, 2,4-dinitrobenzene sulfonic acid; TNBS, 2,4,6-trinitrobenzenesulfonic acid; UC, ulcerative colitis; CD, Crohn's disease; DNCB, Dinitrochlorobenzene.

The presence of such a multitude of mouse models indicates that IBD is mediated by complicated, multifactorial mechanisms. As expected, this complexity is greater in human beings, where environmental and clinical factors, such as smoking, diet, drugs, ethnicity, geographical area, social status, gender, stress and appendectomy, further modulate onset of IBD pathologies [43–46].

3.1. Inhalation studies investigating the effect of CS in rodent models of IBD

Clinical and pre-clinical findings suggested divergent effects of smoking or smoke constituents on the pathophysiology of the gut depending mainly on two conditions, the IBD subtype and the route of administration of the active substance (such as nicotine or CS). Active human smoking is difficult to mimic under laboratory conditions, while classical *in vitro* approaches have translational limitations. Thus, several animal models have been used to assess the impact of CS, nicotine or non-nicotine CS constituents on intestinal pathophysiology [47]. Both genetic- and chemically induced IBD models have been used and effects of various treatment regimens on gut inflammation in these systems are summarised in **Table 2**. There is a general consensus that CS and nicotine administration do not cause macroscopic or histological damage or inflammation in the healthy gut. However, differences in immune cell recruitment [48], cytokine secretion [49–51], mucosal barrier [52, 53] and oxidative stress were observed [54, 55], although without evident tissue damage.

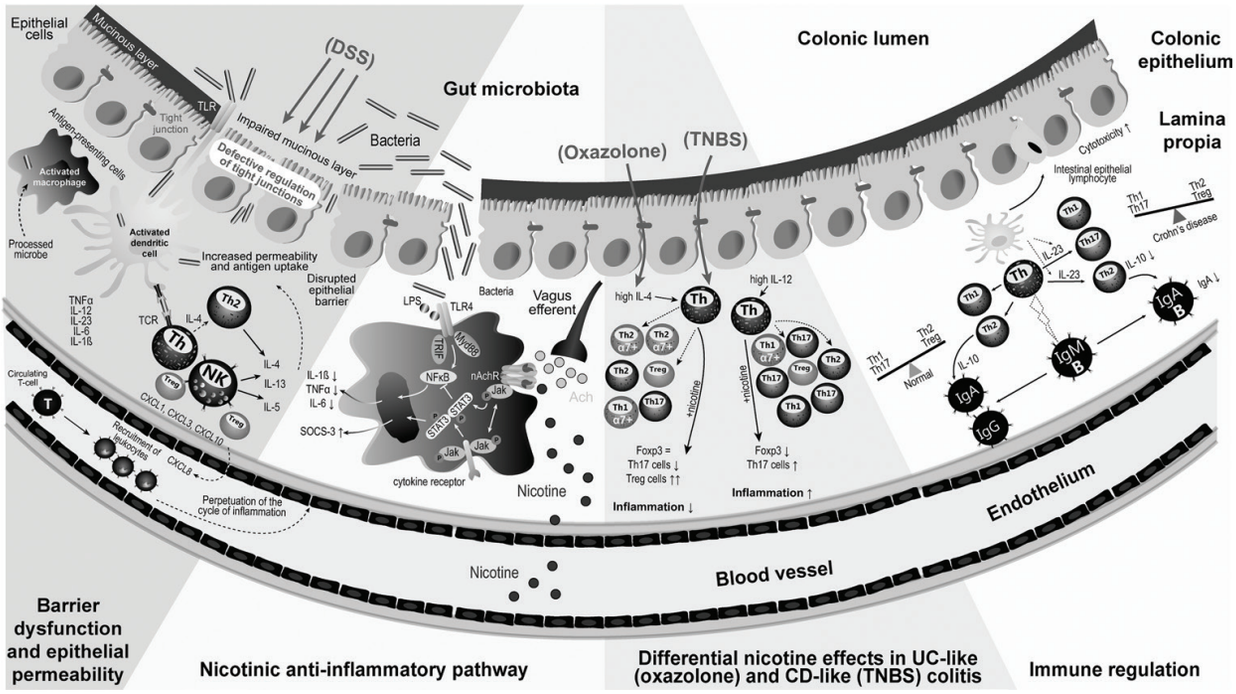

Figure 1. Schematic view of major inflammatory and anti-inflammatory mechanisms implicated in inflammatory bowel diseases and the potential role of a nicotinic anti-inflammatory pathway. Top: altered microbiota in the colonic lumen and/or epithelial-damaging factors (e.g., DSS in experimentally induced colitis) lead to the disruption of the epithelial barrier function and the consequent infiltration of bacteria and other antigens. Middle: various inflammatory processes can be triggered in the lamina propria by the infiltrating bacteria (DSS-induced epithelial barrier; "Barrier dysfunction and epithelial permeability" and "Nicotinic anti-inflammatory pathway" sectors), haptens (oxazolone- and TNBS-induced inflammation, "Differential nicotine effects in UC-like (oxazolone) and CD-like (TNBS) colitis" sector) or by endogenous dysregulation of the balance between Th1/Th17-driven and Th2-driven immune activities, (genetically engineered mouse models; "Immune regulation" section). A hypothetical role of nicotinic receptor-mediated anti-inflammatory response is depicted in the "Nicotinic anti-inflammatory pathway" sector. Bottom: the colonic vasculature is symbolized as a tube running perpendicular to the cross section of the colon. The blood stream delivers leukocytes recruited by cytokine shedding from the local inflammatory sites and enables the perpetuation of the inflammation, e.g., via circulating T-cells. Systemically provided nicotine could increase the anti-inflammatory nicotinic signaling that is naturally transmitted by acetylcholine shed from the efferents of the vagus nerve that innervate the colonic wall. For details of these mechanisms, see Chapter 4.1 to 4.4. Modified from: De Jonge & Ulloah (2007), Ordas et al. (2012), Xu et al. (2014).

Consistent with results of human epidemiological studies, CS had opposing effects on development of CD (negatively) and UC (positively) in several, but not all, of their respective IBD models. Only a few of these studies used inhalation exposure (**Table 2**) and most of their findings mimicked the effects of smoking in humans with IBD.

Thus, the dichotomous effects of CS inhalation, on development of CD versus UC, were perfectly reproduced using two different rat IBD models [54–60]. 2,4,6-trinitrobenzenesulphonic acid (TNBS) and 2,4-dinitrobenzene sulphonic acid (DNBS) were instilled into the rat colon to induce, respectively, CD- and UC-like symptoms. Indeed, pre-exposure of rats to CS increased acute (2–24 h post-induction) intestinal inflammation in the TNBS-induced colitis (CD-like) model [54–57]. The authors used a ventilated smoking chamber filled with a fixed concentration of smoke, delivered by burning commercial cigarettes at a constant rate (2 or 4%, vol/vol, smoke/air) [61]. These results showed that promotion of neutrophil infiltration, as well as free radical production with the accumulation of reactive oxygen metabolites in the intestinal

IBD model	IBD subtype—species	Treatment	Endpoint observed	Effects on intestinal inflammation	References
TNBS colitis	CD—rat	Cigarette smoke (inhalation)	Mucosal damage: ↑ MPO activity: ↑ LTB_4 level: ↑ GSH level: ↓ ROM generation: ↑ TNF-α protein: ↑ SOD activity: ↓ iNOS activity: ↑ COX2 protein: ↑	↑	[54–57]
		Oral nicotine	LTB_4 level: ↓ PGE2 level: = MPO activity: ↓ Histology score: ↓ iNOS protein: ↓ Serum IL-1: =	Low dose: ↓ High dose: ↑ or no effect	[78, 79]
	CD—mouse	Subcutaneous nicotine	Histology score: ↑ DAI scoring: ↑ Treg/Th17 cell ratio: ↓ α7nAChR expression in T cells: no	↑	[77]
		Carbon monoxide (inhalation)	Histology score: ↓ MPO activity: ↓ TNF-α protein and RNA: ↓	↓	[73]
		Oral TCDD	Histology score: ↓ Colon cytokine proteins: ↓ Gene expression Immune cells in MLN and colon	↓	[174]
Iodoacetamide	CD—mouse	Oral nicotine	Mucosal damage: J↑; C↓ iNOS activity: J NA; C= MPO activity: J=; C NA PGE_2 level: J↓; C↓ Histology score: J↑; C↓	Jejunitis: ↑ Colitis: ↓	[175]

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IBD model	IBD subtype—species	Treatment	Endpoint observed	Effects on intestinal inflammation	References
IL-10$^{-/-}$ mice	CD—mouse	Oral nicotine	Mucosal damage: J↑; C↓ Histology score: J↑; C↓ Gene expression	Jejunitis: ↑ Colitis: ↓	[52]
		Carbon monoxide (inhalation)	Histology score: ↓ Colon cytokine proteins: ↓ Gene expression	↓	[71]
DNBS colitis	UC—rat	Cigarette smoke (inhalation)	Histology score: ↑ Mucosal damage: ↑ MPO activity: ↑	↑	[58]
		Subcutaneous nicotine	Mucosal damage: ↓ MPO activity: ↓ LTB_4 level: ↓ ROM generation: ↓ Colon cytokine proteins: ↓	↓	[59]
		Cigarette smoke (inhalation)	Mucosal damage: ↓ MPO activity: ↓ LTB_4 level: ↓ ROM generation: ↓ Colon cytokine proteins: ↓	↓	[59]
		Cigarette smoke (inhalation)	Histology score: ↓ Mucosal damage: ↓ MPO activity: ↓ iNOS activity: ↓ LTB_4 level: ↓ Colon cytokine proteins: ↓	↓	[60]
Oxazolone colitis	UC—mouse	Subcutaneous nicotine	Histology score: ↓ DAI scoring: ↓ Treg/Th17 cell ratio: ↑ α7nAChR expression in T cells	↓	[77]

IBD model	IBD subtype—species	Treatment	Endpoint observed	Effects on intestinal inflammation	References
DSS Colitis	UC—mouse	Oral nicotine	Histology score: ↓ DAI scoring: = MPO activity: = PGE_2 level: ↓	↓	[80]
		Subcutaneous nicotine	DAI scoring: ↓ Histology score: ↓ miRNA expression	↓	[50]
		Cigarette smoke (inhalation)	Colon cytokine RNA: ↓ MPO activity: ↓ Infiltrating immune cells DAI scoring: ↓	↓	[22]
		Cigarette smoke (inhalation)	Mucosal damage: = Colon cell proliferation: = Colon cell apoptosis: = Colon angiogenesis: ↑ Bcl2/VEGF protein: ↑	No effect	[66]
		Subcutaneous nicotine	DAI scoring: ↓ Histology score: ↓ MPO activity: ↓ TNF-α and IL-6 mRNA: ↓	↓	[176]
		Oral nicotine	DAI scoring: ↓ Histology score: ↓ Colon TNF-α protein: ↓ MPO activity: ↓ Colon cytokines mRNA: ↓	↓	[81, 102]
		Oral cotinine	DAI scoring: =	No effect	[81]
		Subcutaneous nicotine	DAI scoring: = Histology score: = Colon TNF-α protein: =	No effect	[81]
		Intraperitoneal nicotine	DAI scoring: = Histology score: ↓ Colon TNF-α protein: ↑	No effect	[81, 82]
		Oral TCDD	Histology score: ↓ Colon TNF-α RNA/protein: ↓ MPO mRNA: ↓	↓	[177]

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IBD model	IBD subtype—species	Treatment	Endpoint observed	Effects on intestinal inflammation	References
TCR$\alpha^{-/-}$ mice	UC—mouse	Carbon monoxide (inhalation)	Histology score: ↓ Colon cytokines RNA/protein: ↓	↓	[72]
Clostridium difficile ToxA	UC—mouse	Intraluminal nicotine	MPO activity: ↓ LTB_4 level: ↓ Luminal fluid: ↓ Substance P release: ↓	↓ Colon; No effect in ileum	[178]

↑, potentiating effect; ↓, attenuating effect; =, no changes; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TCR, T cell receptor; NA, not applicable; ROM, reactive oxygen metabolites; DAI, disease activity index (for further details please see the reference), MPO, myeloperoxidase; LTB4, leukotriene B4; PGE2, prostaglandin E2; SOD, superoxide dismutase 2; COX, cyclooxygenase; iNOS, nitric oxide synthase.

Table 2. Effects of cigarette smoke or related compounds in experimental models of IBD.

tissues, contributed significantly to the potentiating effects of CS on intestinal inflammation. In contrast, in DNBS-treated rats (UC-like model), CS inhalation improved macroscopic signs of colitis at the mucosal level and decreased the levels of colonic pro-inflammatory cytokines [59, 60]. In these latter papers, Ko et al. used a similar inhalation method to the aforementioned study [61], but with a different time of exposure and a few "homemade" modifications to the smoking chamber. One study, conducted in DNBS-treated rats exposed to CS for 15 days before and 2 days after DNBS instillation, showed increased macroscopic and histological damage in the CS-exposed rat colon [58]. Noteworthy, this study used a different inhalation method than did the others. Rats were exposed to a rhythmic inhalation of smoke, with only the nose exposed to the specialized chamber [62], and this chamber was filled with mainstream smoke from a high tar, unfiltered cigarette.

Furthermore, the effect of CS on the development of small intestinal inflammation (CD-like pathophysiology) was studied in a $TNF^{\Delta ARE}$ mouse model [63]. In this mouse model, a knock-in mutation determines the deletion of the AU-region of the TNF-α mRNA, resulting in a systemic TNF-α overproduction and the consequent development of chronic Crohn's-like ileitis and inflammatory arthritis [64]. The authors exposed the mice to CS 4 times a day with 30 min smoke-free intervals, 5 days per week for 2 or 4 weeks [65]. Contrarily to what obtain in human and rat CD, in this model CS did not modulate gut inflammation. Both molecular (e.g. inflammatory and autophagy gene expression) and histopathological endpoints were not affected by CS smoke compared to fresh air exposed mice.

In contrast to its effects in CD rodent models, CS exposure for 2 weeks decreased UC-like inflammation in an acute DSS-induced colitis model in mice [22]. Montbarbon et al. showed a significant decrease in macroscopic and histological colon damage, as well as in colonic pro-inflammatory cytokine expression, in DSS-exposed mice after CS inhalation. Interestingly, this study highlighted a pivotal role for a specific intestinal lymphocyte type, iNKT, in the CS-dependent protection of the colon. The authors used a ventilated smoking chamber of the InExpose® System and exposed the mice to the mainstream smoke of research cigarettes 5 days per week (5 cigarettes/day). However, a previous study, in a long-term mouse model of DSS-mediated chronic colitis, showed a CS-dependent increase in inflammation-associated colon adenoma/adenocarcinoma formation. Although specific inflammatory endpoints were not reported, the number of colon adenomas/adenocarcinomas was significantly increased in the CS-exposed mice [66]. This tumour formation was associated with inhibition of cellular apoptosis and supported by increased angiogenesis. As a possible explanation for this discrepancy, this study used Balb/c mice while the protective effects of CS [22] were observed in C57BL/6 mice. Opposite responses in Balb/c mice, compared with C57BL/6 and other mouse strains, were also reported for other chemical inducers of IBD [67]. Moreover, a different inhalation method was applied in the Balb/c mouse study. These mice were exposed to 2 or 4% CS in a ventilated smoking chamber for 1 h per day.

In the context of inhalation studies aimed to understand the major CS component responsible for the observed anti-inflammatory effects in the intestine, three studies on the anti-inflammatory properties of carbon monoxide (CO) in IBD models are notable. Indeed, CO, a prominent component of CS long considered as just being a toxic gas [68], was recently shown to exert

potent cell protective effects because of its anti-inflammatory, anti-apoptotic and anti-oxidant capabilities [69, 70]. In three different studies, inhaled CO consistently decreased inflammation in chemically induced and genetic mouse models of UC and CD, respectively [71–73]. In particular, the same group of researchers [71, 72] exposed two different knockout mouse models, IL-10$^{-/-}$ [74] and TCR$\alpha^{-/-}$ [75, 76], to CO at a concentration of 250 ppm (part per million) or compressed air (control), attempting to recapitulate, at least in part, CS effects on the development of CD and UC, respectively. IL-10$^{-/-}$ mice were generated by gene targeting in 1993 by Kuhn et al. [74], introducing two stop codons in exon 1 and 3 of the IL-10 gene in murine ES cells. These mice are characterised by extensive Th1-mediated enterocolitis originated by an antigen-driven uncontrolled immune response mainly resembling human CD condition. T cell receptor (TCR) α knockout mice were generated with a similar gene targeting approach [76], thus integrating a neomycin cassette in the first exon of the TCRα locus. In these mutant mice, the intestinal mucosal immunoregulatory mechanisms are negatively affected, triggering the development of UC-like symptoms [75]. Surprisingly, CO inhalation suppressed inflammation in both models, regardless of their IBD subtype, through a heme oxygenase (HO)-1 dependent pathway. The anti-inflammatory capabilities of CO were also confirmed in a TNBS-induced mouse model of CD. Mice were exposed to CO at 200 ppm, beginning after TNBS administration and throughout the remaining study period (3 days) [73]. Thus, the increased colonic damage induced by TNBS was significantly inhibited by the CO treatment, with a consistent suppression of inflammatory markers, such as TNF-α levels and myeloperoxidase (MPO) activity.

As highlighted in the aforementioned reports, although CS or CS component inhalation studies in mouse models seem to recapitulate most epidemiological observations in humans, differences in the inhalation methodologies are many and frequent, making impossible a clear and solid comparison between the studies.

The route of administration was relevant on the final effect also when single CS components, such as nicotine, were administered to IBD mouse models or patients [47]. Thus, in a TNBS mouse model of CD, the detrimental effects of subcutaneous nicotine administration [77] contrasted with the dose-dependent bivalent effect of nicotine administered in the drinking water, that is, positive at low and negative at high concentrations [78, 79]. Furthermore, subcutaneous or oral nicotine administration to rats treated with DNBS led to, respectively, decreased or increased colon inflammation [58, 59]. Finally, while oral or subcutaneous nicotine administration attenuated inflammation caused by DSS treatment in mice [50, 80], intraperitoneal nicotine injection had no effects [81, 82]. Inconsistencies related to different routes of administration of CS components were also observed in human studies [83–86]. Overall, these observations suggested that the route of administration of a CS-related compound, such as nicotine, is important to consider in treating colitis. In animal models, it is clear that mimicking the nicotine intake profiles in smokers (inhalation) could result in increased treatment efficacy. This idea was supported in humans by the conflicting results obtained by local nicotine application (enemas) [87]. Therefore, although the colon may be an important site of action for CS components, the responsible molecule for the observed effects might act on many peripheral and central inflammatory pathways, such as vagus-related anti-inflammatory nicotinic signalling, or might require intermediate metabolic transformations.

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3.2. Limits and pitfalls of studies using inhalation mouse models

Among the aforementioned studies, only a few used inhalation exposure (**Table 2**) models were observed, although many of the findings mimicked human smoking effects in IBD, the results were still variable. Such heterogeneity in observed CS effects on experimentally induced colitis is not unexpected, given variability in animal species and strains, IBD inducers, CS exposure schedules, endpoints and observation periods.

When comparing such quality-relevant exposure conditions, group sizes were usually sufficient, but most of the studies used only male mice or rats, instead of both genders as recommended by the Organisation for Economic Co-operation and Development (OECD) test guidelines. Only one rat study employed the preferable nose-only inhalation mode [58]. Many of the papers did not describe the exposure chambers sufficiently and explanations of exposure concentration parameters (such as number of puffs, flow rate and chamber volume) often did not enable derivation of the standard Total Particulate Matter (TPM) or smoke constituent concentration values, in a weight per volume unit (e.g. mg/L). The most evident heterogeneity among studies, however, was in exposure schedules and durations. The CS inhalation studies in IBD models typically used daily exposure durations no longer than one hour, with none using the recommended 6 h/day duration. Some studies pre-exposed the animals a few days before IBD induction and discontinued CS exposure after the induction treatment, while others continued exposure until the end of the study or began CS inhalation after IBD induction [59]. To explore more systematically the effects of inhaled CS or CS constituents on IBD in various models, there is a clear need to harmonise exposure conditions to be closer to minimal standards for inhalation toxicity studies. This is particularly true for exposure schedules and durations, as well as for documentation of meaningful concentration measurements in the exposure atmospheres (**Table 3**). Finally, to elucidate the molecular mechanisms of IBD-CS interactions, beyond the current knowledge, it will be necessary to combine robust IBD models (UC and CD), well-controlled, state-of-the-art inhalation exposure design and technology and disease-specific endpoints with systems-wide molecular profiling. We conducted systems toxicology-oriented inhalation studies using mouse models to investigate effects of CS and candidate modified risk tobacco products in chronic obstructive and cardiovascular diseases [33, 88–91]. These studies demonstrated the feasibility and suitability of this approach for identifying the molecular basis of disease mechanisms and the biological impacts of CS. The study design and inhalation exposure technology were based on the OECD guidelines TG412 and TG413 for 28 and 90 days inhalation toxicity studies, respectively [92, 93]. Satellite groups were included to provide material for the additional molecular investigations and a similar study was conducted on rats exposed to nicotine aerosols [33]. A very detailed description of the study design and methodology was provided [94] and this might serve as a template for new IBD inhalation studies. Of course, adaptations will be necessary, based on specifications of the IBD models. For example, most chemically induced IBD models require acute, rather than subchronic or chronic, observation periods, while the genetically engineered IBD models develop the disease in a similar timeframe as the COPD and CVD models.

IBD model, induction	Study design	Exposure duration	Inhalation technology	CS/inhalant characterisation	References
(OECD TG 412 recommendation)	At least 5 males and 5 females per group, 3 dose levels of test article, filtered air and/or vehicle control	6 h/day; 5 (7) days/ week; 28 days	Nose-only preferred, whole body acceptable, detailed description of exposure chamber to be given	Analytical characterisation; respirable particle size (1–3 μm MMAD), nominal and actual test article concentration (mass per volume) to be indicated, constant concentration during exposure period	[92]
Rat (Sprague Dawley), TNBS enema	8–10 rats/group (males only), 1 dose level, fresh air control	1 h/day; 4 days pre-induction	Whole body, ventilated smoking chamber (20 L) with 5–6 rats, smoke generated with peristaltic pump	"Camel" cigarettes, 4% v/v smoke, no characterisation	[56]
Rat (Sprague Dawley), TNBS enema	10–12 rats/group (males only), 2 dose levels, fresh air control	1 h/day; 4 days pre-induction	Whole body, ventilated smoking chamber (20 L) with 5–6 rats, smoke generated with peristaltic pump	"Camel" cigarettes, 2 and 4% v/v smoke, no characterisation	[55]
Rat (Sprague Dawley), TNBS enema	6–8 rats/group (males only), 1 dose level, fresh air control	1 h/day; 4 days pre-induction	Whole body, ventilated smoking chamber (20 L) with 5–6 rats, smoke generated with peristaltic pump	"Camel" cigarettes, 4% v/v smoke, no characterisation	[54]
Rat (Sprague Dawley), TNBS enema	10 rats/group (males only), 2 dose levels, fresh air control	1 h/day; 8 days pre-induction	Whole body, ventilated smoking chamber (20 L) with 5–6 rats, smoke generated with peristaltic pump	"Camel" cigarettes, 2 and 4% v/v smoke no characterisation	[57]
Mouse (C57BL/6), DSS in drinking water	6–10 mice/group (males only), 1 dose level, fresh air control	2 week (5 days/ week) pre-induction and 1 week post-induction	Whole body, InExpose chamber (Scireq) and rotary smoking machine	3R4F reference cigarettes, mainstream smoke from 5 cigarettes (8 puffs per cigarette), no concentration/ characterisation	[22]
Rat (Sprague Dawley), DNBS enema	6–8 rats/group, 3 dose levels, fresh air control (10 rats/group)	5–40 min/day, 15 days pre-induction and 2 day post-induction	Nose-only, puffwise smoke injection into chamber	2R1 reference cigarette, 5, 20 or 40 puffs/day (undiluted), no concentration/ characterisation	[58]

IBD model, induction	Study design	Exposure duration	Inhalation technology	CS/inhalant characterisation	References
Rat (Sprague Dawley), DNBS enema	7 rats/group, 2 dose levels, fresh air control	1 h/day; 3 days post-induction	Whole body, ventilated smoking chamber (20 L) with 5–6 rats, smoke generated with peristaltic pump smoke, no characterisation	"Kings" cigarettes, 4% v/v; no concentration/ characterisation	[59]
Rat (Sprague Dawley), DNBS enema	6–8 rats/group, 1 dose level, fresh air control	1 h/day; 3 days pre-induction, 4 day post-induction	Whole body, ventilated smoking chamber (20 L) with 5–6 rats, smoke generated with peristaltic pump	"Camel" cigarettes, 2 and 4% v/v smoke, no characterisation	[60]
Mouse (Balb/c), DSS in drinking water	5–12 mice/group (males only), 2 dose levels, fresh air control	3 cycles of: 7 days DSS + CS (1 h/day) followed by 14 days recovery	Whole body, ventilated smoking chamber (20 L), smoke generated with peristaltic pump	"Camel" cigarettes, 2 and 4% v/v smoke, no characterisation	[66]
TCR$\alpha^{-/-}$ mouse (C57BL/6)	10 mice/group (5 males and 5 females), 1 dose level, fresh air control	4 week (daily duration not indicated)	Whole body, 3.70 ft^2 plexiglass animal chamber, 12 L/min flow rate	CO gas, 250 ppm in air, continuous measurement	[72]
IL-10$^{-/-}$ mouse (C57BL/6)	12 mice/group (males only), 1 dose level, fresh air control	4 week (daily duration not indicated)	Whole body, 3.70 ft^2 plexiglass animal chamber, 12 L/min flow rate	CO gas, 250 ppm in air, continuous measurement	[71]
Mouse (C57BL/6), TNBS enema	12 mice/group, 1 dose level, fresh air control	3 day (permanent) post-induction	Whole body, acrylic chamber	CO gas, 200 ppm in air, continuous measurement	[73]

Table 3. Comparison of exposure conditions in published inhalation studies using rodent IBD models.

4. Mechanisms of IBD pathogenesis with possible relationship to CS constituents

4.1. Nicotinic anti-inflammatory pathway

The vagus nerve transmits signals by releasing acetylcholine that, in turn, stimulates neuronal and immune cells via their nicotinic acetylcholine receptors (nAChR) [95, 96]. These are ligand-gated ion channels expressed not only in neuronal cells, but also in most mammalian non-neuronal cell types, though different cell type-specific downstream signalling functions [97]. In the nicotinic anti-inflammatory pathway, nAChR activation by acetylcholine or other ligands inhibits the downstream NF-κB pathway, attenuating production of TNF-α and other

cytokines [98, 99]. This pathway was reported to be one of the most likely explanations for CS-associated anti-inflammatory responses in the gut. Mapping the relevant neuronal circuits revealed that efferent vagus nerve fibres innervated the small intestine and proximal colon [100]. Vagotomised mice were more susceptible than normal mice to developing colitis after exposure to DSS and had increased levels of NF-κB and cytokines, such as IL-1β, IL-6 and TNF-α [101–103]. Pretreatment with nicotine reversed these effects through activation of α7nAChR, identified as the major receptor involved in nicotinic anti-inflammatory pathways [99, 104]. Potential therapeutic applications of selective α7nAChR agonists, such as the partial α7 agonists 3-(2,4-dimethoxybenzylidene)-anabaseine (GTS-21) and anatabine citrate, and of α7nAChR-positive allosteric modulators, was explored in pre-clinical and clinical studies [105–109]. Moreover, additional nAChR subtypes, such as α4β2, α3β4, α3β2 and α6, were also proposed as targets for nicotine treatment [110–112], increasing the complexity, but also the therapeutic potential, of this approach. Although research on the mechanisms involved in nicotinic anti-inflammatory pathways has highlighted the pharmacological potential of nAChR agonists, studies showing contradictory results obtained with specific α7nAChR ligands [82] suggested that these compounds should be used with caution in patients with IBD.

4.2. Immune regulation

The immunosuppressive effects of cigarette smoking, on both cellular and humoral immunity, have long been recognised [113–115]. Studies exploring how nicotine or CS can suppress the immune system indicated that, in nicotine-treated animals, T cells did not enter the cell cycle and proliferate as expected. Similar effects were observed in smokers and in animals exposed to CS [116–118]. Several studies described the implications of CS for different immune cell types, as well as the diverse actions of nicotine or CS, depending on the pathological environment, for example, UC or CD, in which the immune cells originated [77, 99, 112, 119–122]. For instance, when stimulated by lipopolysaccharide, peripheral blood mononuclear cells derived from smokers showed decreased IL-8 release only if subjects were also CD patients [122]. Similarly, the same investigators demonstrated that smokers with CD had significantly lower IL-10 (anti-inflammatory)/IL-12 (pro-inflammatory) ratios than non-smokers or smokers with UC. As suggested in some reports, the differential signalling of dendritic cells from CD (Th1-like) and UC patients exposed to cigarette smoke extract (CSE) *in vitro* could play a role in the opposing responses of cigarette smoke exposure, that is, a Th1-like response in CD, with increased Foxp3-positive CD4 T cells [121].

4.3. Barrier dysfunction and intestinal permeability

The intestinal mucosa is one of the most important physical barriers against external threats. Changes in intestinal permeability are crucial for the development of IBD [123] and several studies implicated CS in regulating barrier integrity. However, the effects of smoking on intestinal permeability are controversial. Several *in vitro* and *in vivo* observations, in studies using humans or rodents, suggested that decreased intestinal permeability in smokers might explain the protective effects of smoking in UC [53, 124–127]. In contrast, a recent article reported that mice exposed to CS exhibited increased intestinal permeability and bacterial translocation, intestinal villi atrophy, damaged tight junctions and abnormal tight junction

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proteins [128]. However, no intestinal barrier changes were identified in the colons of control or CS-exposed mice, suggesting that there was CS-related organ specificity and, thus, possibly explaining the opposing effects of smoking on CD and UC.

4.4. Gut microbiota

Much evidence supports the strong impact of environmental factors on gut microbiota, and smoking has recently been investigated as a potential factor shaping the microbiota. This potential connection implied new possibilities regarding the role of smoking in IBD development. Thus, studies targeting selected bacterial groups reported that patients with active CD, who also smoked, had microbial profiles different from those of non-smoking patients with CD. Similar results were found in healthy smoking controls, suggesting that the association related not to intestinal inflammation but, instead, to a direct impacts of smoking on the microbiota [129, 130]. Differences between mice and humans at the level of the gut microbiota limit the usefulness of mouse models, relevant to CS, gut microbiota and IBD. However, a few studies using rats and mice were consistent with observations in humans, indicating CS-dependent shifts in gut microbiota compositions [131–133]. These observations supported a possible role for CS in shaping the gut microbiome, with potential, though still unknown, consequences for evolution of inflammation-related disorders, such as IBD.

4.5. Other potential mechanisms

Currently, the processes described in Sections 4.1–4.4 have been those most explored as potential links between CS and IBD development. However, there are several other possible mechanisms, indicative of how environmental factors might exponentially increase complexity of IBD pathology.

4.5.1. Colon motility

In UC, fasting colonic motility increased, whereas motor responses to food significantly decreased [134]. Observations in experimental animals and humans showed that nicotine promoted smooth muscle relaxation, reducing symptoms, such as diarrhoea and urgency without significantly influencing inflammation [135–137].

4.5.2. Eicosanoid-mediated inflammation

Smoking and nicotine may also affect UC by reducing eicosanoid-mediated inflammatory responses. Two studies independently demonstrated this specific effect in humans and rabbits [53, 138].

4.5.3. Rectal blood flow

Patients with UC have significantly higher rectal blood flow than normal controls, but smoking decreased rectal blood flow to within normal ranges [139–141]. However, changes in blood flow can affect intestinal inflammation in opposing ways. Decreasing blood flow can reduce levels of inflammatory mediators that reach the mucosal surface, while long-term impairment

of rectal mucosal microvascular blood flow can result in a higher incidence of anastomotic breakdown in chronic smokers [140].

4.5.4. Non-nicotine-mediated effects

Although nicotine is considered to be the major mediator of CS effects on intestinal inflammation, there is a clear evidence for involvement of other smoke constituents in CS-dependent responses. Both UC and CD mouse models were affected by carbon monoxide (CO) inhalation [71–73, 142]. These studies suggested that the mechanism through which CO protected against intestinal inflammation involved promoting bactericidal activities of macrophages [142]. Nitric oxide (NO) was also suggested as contributing to beneficial CS effects, based on its relaxant effects on colonic smooth muscle from UC patients [143]. Moreover, physiological NO, derived from nicotine-stimulated intestinal neuronal cells, functioned as a mediator in smooth muscle relaxation in the colons of DSS-treated mice [137].

5. Conclusions

Smoking cigarettes is addictive and causes a number of serious diseases, including those of the respiratory and cardiovascular system [144], it also negatively impact on the gastrointestinal tract, such as CD [145]. Many of the adverse health effects of smoking are reversible and important health benefits are associated with smoking cessation [146]. With regard to the other major IBD form, a protective effect of cigarette smoking on the risk of UC development is well documented. However, whether CS constituents have beneficial effects on the course of the disease is less clear and the potential mechanisms are not understood.

CS inhalation studies in IBD mouse models would, ideally, reproduce the clinical effects of CS on colonic inflammation. This would facilitate identification of the mechanisms involved in the effects of CS on colitis and, eventually, lead to the characterisation of new anti-inflammatory processes involved in colon protection [22]. Nonetheless, so far, the results obtained using animal models of IBD following exposure to inhaled CS or to nicotine via non-inhalation routes, reflected the ambiguity of the clinical observations. These inconsistencies often reflect the high variability related to animal models (e.g. strains, IBD inducers, etc.) and inhalation methodologies. A more systematic and standardised approach is required to obtain consistent and reproducible data addressing the mechanisms by which CS interacts with the inflammatory processes in animal models of UC-like and CD-like colitis. Such systematic investigations could provide valuable insights into the possible anti-inflammatory effects of CS constituents in models related to UC. Corresponding studies in CD models would provide more mechanistic detail about how these compounds can enhance inflammation in CD.

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Conflict of interest

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