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Chemistry meets biology in colitis-associated carcinogenesis

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Abstract

The intestine comprises an exceptional venue for a dynamic and complex interplay of numerous chemical and biological processes. Here, multiple chemical and biological systems, including the intestinal tissue itself, its associated immune system, the gut microbiota, xenobiotics, and metabolites meet and interact to form a sophisticated and tightly regulated state of tissue homeostasis. Disturbance of this homeostasis can cause inflammatory bowel disease (IBD) – a chronic disease of multifactorial etiology that is strongly associated with increased risk for cancer development. This review addresses recent developments in research into chemical and biological mechanisms underlying the etiology of inflammation-induced colon cancer. Beginning with a general overview of reactive chemical species generated during colonic inflammation, the mechanistic interplay between chemical and biological mediators of inflammation, the role of genetic toxicology and microbial pathogenesis in disease development are discussed. When possible, we systematically compare evidence from studies utilizing human IBD patients with experimental investigations in mice. The comparison reveals that many strong pathological and mechanistic correlates exist between mouse models of colitis-associated cancer, and the clinically relevant situation in humans. We also summarize several emerging issues in the field, such as the carcinogenic potential of novel inflammation-related DNA adducts and genotoxic microbial factors, the systemic dimension of inflammation-induced genotoxicity, and the complex role of genome maintenance mechanisms during these processes. Taken together, current evidence points to the induction of genetic and epigenetic alterations by chemical and biological inflammatory stimuli ultimately leading to cancer formation.

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Keywords

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1. Introduction: inflammatory bowel disease and colon cancer

With respect to a functional interplay between chemistry and biology, the intestinal tract is one of the most complex organs in the body. In the intestine, local epithelial cells closely interact with the body's immune system and meet with a complex bacterial microflora and a plethora of endogenous metabolites and xenobiotic substances. All of these factors need to be tightly balanced to ensure intestinal homeostasis and to guarantee efficient uptake of nutrients and excretion of metabolites. If this balance is disturbed, pathological states can appear, such as inflammatory bowel disease (IBD). As a major risk factor for colon cancer, IBD entails chronic, relapsing inflammation of the gastrointestinal tract that affects millions of people worldwide, with >1 million new patients each year in the US alone [1]. The paucity of safe and effective therapies for IBD amplifies its public health impact and motivates the hunt for the molecular etiology of the disease and the mechanisms linking colitis with colon carcinogenesis. This review addresses recent developments in chemical and biological mechanisms underlying inflammation-induced colon cancer, starting with a general overview of the reactive chemical species generated during colonic inflammation followed by a discussion of the mechanistic interplay between chemical and biological mediators of inflammation and the role of microbial pathogenesis and genetic toxicology in the etiology of inflammation-induced colon cancer. The reader is referred to several recent reviews of molecular aspects of IBD and cancer development not covered here, in particular immunological and genetic studies [1–7].

1.1 Inflammatory bowel disease: Crohn's disease and ulcerative colitis

Inflammatory bowel disease comes in two distinct forms: Crohn's disease and ulcerative colitis. In Crohn's disease, inflammation often affects the entire thickness of the bowel wall and affects mainly the ileum and colon, but can discontinuously spread throughout any part of the intestine. In contrast, the inflammation in ulcerative colitis is usually confined to the mucosal surface of the intestines and limited to colon and rectum, spreading in a continuous fashion (reviewed in [1]). The pathophysiology of IBD is multifactorial, with contributions from genetic, epigenetic, environmental, and endogenous microbial factors (reviewed in [3,8]). Genetic background is an important determinant for IBD, evidenced by the fact that family history is a risk factor for disease development with a concordance rate in monozygotic twins of 10–15% in ulcerative colitis and 30–35% in Crohn's disease. At present, more than 150 genetic loci have been associated with disease development [9]. IBD-associated genes are involved in several disease-relevant pathways crucial for intestinal homeostasis, including epithelial barrier function, microbial defense mechanisms, regulation of immunity, autophagy, generation of reactive oxygen and nitrogen species (ROS, RNS) and signaling functions. About 30% of these loci are shared between ulcerative colitis and Crohn's disease, so the two pathological states share some similar molecular mechanisms, but also exhibit many disease-specific features [3,9]. The contribution of environmental

factors is not well understood, but evidence indicates that they play a significant role, as suggested by the high prevalence of IBD in industrialized countries. This pattern of high prevalence appears not to be related to genetic factors, since migrant studies have demonstrated that individuals relocating from a region with low prevalence to one of high prevalence are at increased risk of developing IBD [10]. Environmental influences include life-style factors such as smoking and dietary composition among others, but may also be related to improved sanitation and hygiene. The latter is based on the hypothesis that lack of childhood exposure to enteric pathogens may lead to inappropriate immune responses to new antigens in adulthood and an imbalance in host-microbe interaction, which play an important role in the maintenance of mucosal homeostasis [10]. An imbalance of host-microbe homeostasis is of paramount importance in the etiology of IBD, supported by the fact that some IBD patients benefit from antibiotic treatment and most mouse models of IBD require the presence of intestinal bacteria to develop colitis [1]. Furthermore, an altered immune response to the intestinal flora plays a pivotal role in the etiology of IBD [8]. Notably, the intestine and its associated tissues represent the largest immunological organ of the body. In particular, the intestinal *lamina propria* contains a complex mixture of immune cells that maintain immune tolerance and pathogen defense in a tightly controlled balance (reviewed in [1]). Active IBD is characterized by a massive infiltration of innate immune cells, including neutrophils, macrophages, dendritic cells, and natural killer cells, which fulfill complex roles under physiological as well as pathophysiological conditions. Importantly in the context of this review, activated neutrophils and macrophages are major sources of ROS and RNS that are among the main factors against invading pathogens, but can also cause collateral damage to the host tissue, thereby potentially initiating and promoting carcinogenesis (see below). Apart from innate immune cells, those of the adaptive immune system (e.g., B and T cells), also control intestinal homeostasis. Perturbation of the balance of Th17 cells, which produce the pro-inflammatory signature cytokines IL-17, IL-21, and IL-23, and regulatory T-cells, which produce the anti-inflammatory cytokines IL-10 and TGF- β appear to be of particular importance. An imbalance in these systems seems to play a crucial role in the etiology of IBD (reviewed in [2]). For an in-depth discussion on the intestinal immunology in the pathology of IBD, the reader is referred to several recent reviews [2,4,6,11–17]. In summary, IBD is a multifactorial disease that is thought to result from a perturbation in host-microbe interactions leading to an immunological imbalance and chronic inflammation in genetically susceptible individuals [3].

Strong epidemiological evidence indicates that inflammation existing in Crohn's disease and ulcerative colitis is associated with increased risk of colon cancer, but the responsible molecular mechanisms remain largely undefined. More than 20% of patients with IBD develop colitis-associated cancers within 30 years of disease onset, and >50% of these patients die from them [7]. Notably, patients who develop IBD at a young age (< 30 yrs) have a much greater risk of cancer development [18]. In general, the risk for colon cancer increases with duration and severity of disease, whereas it decreases when patients are treated with anti-inflammatory drugs such as mesalamine and corticosteroids, consistent with a causative role for inflammation in colon carcinogenesis (reviewed in [19]).

Spontaneous colorectal cancer shares many common pathophysiological mechanisms with colitis-associated cancer, but differs in some distinct ways (reviewed in [7]). For example, both types of cancer progress through a sequence of aberrant crypt foci, polyps, adenomas, and carcinomas. However, dysplasia in spontaneous colorectal cancer is often focal, whereas colitis-associated cancers often develop through a sequence of chronic inflammation, tissue injury, and multifocal dysplasia leading to the formation of poorly-defined carcinomas. The genetic alterations such as chromosomal instability, microsatellite instability, and DNA hypermethylation found in spontaneous colon cancers also occur in colitis-associated cancers; these characteristics arise in inflamed tissue before the appearance of histological evidence of dysplasia or cancer [19]. Common tumor-related signaling pathways, including factors such as Wnt, β -catenin, K-ras, p53, TGF- β , and DNA repair, are affected in development of spontaneous as well as colitis-associated cancers, but at different stages of tumor formation [7]. It is very likely that crypt stem cells represent the cells-of-origin for cancer development, in both spontaneous colorectal and colitis-associated cancers [20]. However, recent studies demonstrate that NF- κ B signaling can lead to dedifferentiation of intestinal epithelial cells into tumor-initiating cells with stem cell-like properties [21]. This is consistent with the fact that colitis-associated carcinogenesis (CAC) is characterized by production of NF- κ B and Stat3-dependent pro-inflammatory cytokines, and activation or inactivation of oncogenes and tumor suppressors, respectively. Reactive chemical species are likely to play an important role in these processes and there is increasing experimental evidence supporting the hypothesis that during inflammation, cells of the innate immune system generate large amounts of ROS and RNS inducing genetic and epigenetic changes, which may lead to mutations and tumor initiation and promotion [22].

1.2 Mouse models of colitis-associated carcinogenesis

Several mouse models of colitis have been developed to study molecular disease mechanisms and to define targets for translational approaches. Colitis is induced by chemical agents, by genetic intervention, by infection or by combinations of these factors (Table 1). In light of the recent criticism of mouse models of human disease [23,24], which in our opinion has been overstated and inaccurate, we demonstrate that many compelling pathological and mechanistic correlates exist between mouse models of colitis-associated cancer and the clinically relevant disease in humans. Indeed, as we review here, a plethora of studies in mouse models of colitis have revealed that many chemical and biological mechanisms of pathology share strong similarities across species boundaries (Table 2), which is most evident by the fact that human IBD and various forms of colitis in mice lead to an increased risk of colon carcinogenesis. Only through the combination of animal models and studies utilizing human IBD patients can a deeper understanding be obtained of the molecular mechanisms in CAC, through the use of multidisciplinary approaches from genetics to microbiology, chemistry, systems biology and mathematical modeling, combined with thoughtful data interpretation.

We recently conducted a systematic analysis of mechanisms and biomarkers of *Helicobacter hepaticus* (*H. hepaticus*)-induced colitis in immunodeficient 129 *Rag2*^{-/-} mice with respect to various aspects of disease development [25–29]. The gram-negative spiral bacterium *H. hepaticus* colonizes the liver and intestinal crypts of the cecum and the colon, establishing a

life-long infection [25,26,30,31]. *H. hepaticus* does not typically cause disease in immunocompetent mice, but infection in susceptible mouse strains or in immunodeficient mice, such as those lacking the recombinaase-activating gene-2 (*Rag2*), results in chronic colitis and colon cancer [25] (Figure 1). *H. hepaticus* infection in *Rag2*^{-/-} mice emulates many aspects of human IBD and as discussed here, this mouse model has proved to be a valuable tool specifically to study features of innate immunity during CAC.

2 Immunological chemistry of inflammatory bowel disease

Colonic inflammation results in a local release of cytokines and other chemotactic factors that cause infiltration and activation of innate immune cells to produce large quantities of other cytokines and chemokines resulting in the activation of various enzymes that generate a wide spectrum of reactive chemical species as summarized in Figure 2 and discussed in the following sections in detail. In particular, we summarize evidence that these chemical mediators of inflammation can react with all types of macromolecules, including proteins, RNA, and DNA, to cause mutagenic and cytotoxic damage, thereby contributing to colon carcinogenesis.

2.1 Cell types involved in the generation of reactive chemical species during IBD

In addition to the endogenous production of NO by colonic epithelial cells [32–35], macrophages and neutrophils are thought to be responsible for the bulk of ROS and RNS generation during acute and chronic inflammation (Figure 2). Both cell types are closely related and cooperate during the onset, progression and resolution of inflammation. Usually, tissue-resident macrophages and dendritic cells sense inflammatory stimuli, which causes chemokine-dependent recruitment of neutrophils followed by infiltration of blood monocytes, both of which then differentiate and become activated (reviewed in [14]).

Initial support of the hypothesis that innate immune cells can act as carcinogens was obtained by Weitzman and colleagues, who exposed mouse fibroblasts to activated human neutrophils or a cell-free ROS-producing system. Following injection of the fibroblasts into nude mice, tumors developed, but only in mice injected with exposed cells. Mice injected with control cells remained tumor-free, which indicated that activated neutrophils and ROS are able to mediate malignant cell transformation [36]. It is well established that neutrophils and macrophages play a pivotal role in the induction and maintenance of inflammation during colitis in mice and humans [26,37–39]. In addition, several studies support the functional relevance of these cell types during colon carcinogenesis. For example, in a rat model of dextran sodium sulfate (DSS)-induced acute colitis, selective depletion of neutrophils by intraperitoneal injection of a neutrophil-specific monoclonal antibody significantly reduced pathology [40]. A similar antibody-based approach was used to selectively deplete mature neutrophils in *H. hepaticus*-infected *Rag2*^{-/-} mice, a model in which there is massive infiltration of macrophages and neutrophils during disease development [26,27,29]. Interestingly, depletion of neutrophils in these mice significantly decreased the severity of colon pathology, including cancer rates, and led to lower expression of pro-inflammatory cytokines and ROS/RNS-generating enzymes (see below) [26]. Macrophages can also contribute to disease progression and severity, as shown in a mouse model of *H. bilis*-induced typhlocolitis, in which macrophages were selectively

depleted by treatment with clodronate-containing liposomes. Clodronate-treated mice exhibited significantly lower histopathology scores and suppressed expression of macrophage-related factors, such as TNF- α , IL-1 β , and iNOS, suggesting that macrophages are important mediators of *H. bilis*-induced colitis. However, the finding that clodronate treatment concomitantly reduced the number of MPO-positive neutrophils suggests that both macrophages and neutrophils closely interact in disease development on a functional level [41]. This view is supported by the *H. hepaticus Rag2*^{-/-} mouse model that revealed cytokine signatures suggestive of both neutrophil and macrophage activity [29]. Interestingly, this finding correlated well with results from patients with Crohn's disease. In contrast, cytokine signatures of patients with ulcerative colitis were more suggestive of neutrophil activity only, indicating specific differences between the two types of IBD on a molecular level [29].

2.2 NADPH oxidases, xanthine oxidoreductase, and the generation of ROS

Superoxide (O₂^{•-}), is the primary source of ROS in humans. Under physiological conditions, mitochondrial respiration is the major source, whereas NADPH oxidase (official gene symbol: *Nox*, not to be confused with NO_x which refers to NO oxidation products) and xanthine oxidoreductase (XOR) significantly contribute to the generation of O₂^{•-} under pathophysiological conditions (reviewed in [42–44]) (Figure 2).

NADPH oxidases represent a widely distributed family of enzymes, with each species consisting of a flavoprotein, cytochrome b and several regulatory subunits. When activated, the enzymes assemble in the cell membrane and reduce oxygen to O₂^{•-}. Phagocytic cells express NADPH oxidase 2 in the phagosomal membrane, where it generates high fluxes of O₂^{•-} (reviewed in [42,43,45]). In addition to 'phagocyte NADPH oxidases 2', NADPH oxidase 1 is highly expressed in the gastrointestinal tract. Levels of the mRNA of NADPH oxidase 1, also known as the 'colon NADPH oxidase', increase from proximal to distal colon [45]. Even under physiological conditions, NADPH oxidase 1 is highly expressed in colon epithelial cells, both within the crypts and on the luminal surface [46,47]. Maturation-dependent factors seem to regulate the expression of NADPH oxidase 1 in colonic epithelial cells, which leads to the highest expression levels in surface mucous cells where NADPH oxidases are thought to contribute to host defense. However, under pathophysiological conditions, they may mediate tissue damage by generating ROS. This view is consistent with results from *H. hepaticus*-induced colitis in *Rag2*^{-/-} mice, in which gene expression of NADPH oxidases 1 and 2 (*Nox 1*, *Nox2*) was significantly up-regulated in inflamed tissue. This was accompanied by higher expression levels of several regulatory subunits of the NADPH oxidase system, such as *Ncf1* (p47phox) and *Ncf2* (p67phox) [27], presumably a result of tissue infiltration of innate immune cells. In addition, studies in mice and human cells have shown that pro-inflammatory cytokines induced during colitis activate expression of NADPH oxidases [48,49]. The role of NADPH oxidase in human IBD and colon carcinogenesis is not well characterized. Some evidence comes from patients with chronic granulomatous disease, many of whom have genetic defects in the NADPH oxidase system that results in an immunodeficiency phenotype characterized by an inability of phagocytes to kill invading pathogens. These patients exhibit an increased risk for IBD, suggesting that NADPH oxidases have a direct role in controlling intestinal homeostasis [45,50,51]. Szanto

et al. reported strong expression of NADPH oxidase 1 in active lesions from patients with Crohn's disease and ulcerative colitis [46] and Fukuyama *et al.* reported significant up-regulation of NADPH oxidase 1 in colonic tumor tissue [47], suggesting a functional role of NADPH oxidases in disease progression. Despite these findings, comprehensive understanding of the functional role of NADPH oxidases in CAC is incomplete, and conclusions rely mainly on correlative studies, while mechanistic studies in mice and humans are limited. Genetic studies analyzing mechanisms of CAC in mice carrying mutations or deletions in the NADPH oxidase system would be of interest with respect to pharmacological interventions, since NADPH oxidases are already being considered as potential drug targets for treatment of other inflammatory disorders, such as vascular disease [43].

Xanthine oxidoreductase (XOR) is a multifunctional enzyme that is expressed and secreted by many epithelial cells including those in the colonic mucosa (reviewed in [44]) (Figure 2). The enzyme possesses two activities, as both a reductase and an oxidase. The reductase catalyzes the oxidative hydroxylation of hypoxanthine to xanthine and subsequently of xanthine to uric acid. In this functional state, xanthine reductase acts as a housekeeping enzyme with a role in purine catabolism, detoxification and the regulation of the cellular redox potential. Various stimuli induce the conversion into an oxidase, either reversibly by thiol oxidation or irreversibly by limited proteolysis. In contrast to the reductase, xanthine oxidase is associated with production of large amounts of ROS including $O_2^{\bullet-}$ [44]. The enzyme has also been directly implicated in the activation of macrophages through effects on chemokine expression and other factors, thereby participating in macrophage-mediated neutrophil recruitment [52].

In the human colon, XOR immunostaining follows a gradient along the villus-crypt axis in a pattern similar to that observed for NOX1, with luminal epithelial cells showing the strongest staining. XOR staining was also observed in cells at the base of the crypt, as well as in neutrophils and endothelial cells in the *lamina propria* [53]. Data on the role of XOR in colitis and associated cancer development is limited and controversial. *H. hepaticus*-infected *Rag2*^{-/-} mice showed higher expression levels of XOR mRNA in inflamed tissue, but the origin of this increase is currently unknown [27]. Up-regulation of XOR is consistent with the finding that XOR is stimulated by IFN- γ , TNF- α , and IL-1, and some of these factors also trigger the reductase-oxidase conversion [44]. Furthermore, Siems *et al.* reported that the XOR inhibitor oxypurinol protects mice from morphological and molecular changes occurring in TNBS-induced colitis. However, as is the case with every pharmacological inhibitor, specificity of the compound is an issue. Although these results indicate a role of XOR in murine colitis and XOR has been associated with other inflammatory diseases [44], its role in human IBD awaits further clarification. Kruidenier *et al.* reported that epithelial apoptosis in patients with Crohn's disease was strongly associated with XOR expression, suggesting a role for XOR in the regulation of epithelial homeostasis. However, in this study, overall levels of XOR were unaffected during intestinal inflammation, indicating that XOR contributes to disease development more through local and functional changes in its activity [53]. Mouse genetics could shed some light on the role of XOR in colitis, which could be instrumental to test potential treatment options by

targeting this enzyme. In this respect, generation of colon-specific knock-out mice or mice that specifically lack the enzymes' oxidase function may represent one possible line of investigation, since *Xor*^{-/-} mice die within the first six weeks after birth due to renal failure [54,55].

Superoxide can be transformed by superoxide dismutases (SOD) into hydrogen peroxide (H₂O₂), which can be either detoxified by catalases and peroxidases into H₂O and O₂, or it can be reduced by divalent metal ions in Fenton chemistry that gives rise to highly reactive hydroxyl radicals ([•]OH) [42] (Figure 2). Since H₂O₂ reacts poorly with most biological molecules and is highly diffusible, it acts as an important signal transducer (reviewed in [42,56]). For example, H₂O₂ activates phosphorylation cascades resulting in downstream effects such as enhanced stress resistance, cell proliferation, cytokine release, cell adhesion, growth arrest and apoptosis. Its damaging effects are mainly caused by its reaction with seleno-, thiol or heme peroxidases to produce radical and non-radical species as well as through [•]OH formed by Fenton chemistry [42]. In mice with TNBS-induced colitis, H₂O₂ levels immediately increased in the *muscularis externa* within the first week after treatment, with peak levels achieved after the first day [57]. That the generation of H₂O₂ acts as a detoxifying pathway is suggested by studies of SOD, the major source of H₂O₂. For example, in DSS-induced colitis, Cu/Zn-SOD-overexpressing transgenic mice showed a survival benefit that was accompanied by an enhanced clearance of neutrophils [58]. Moreover, studies in which SOD and SOD mimetics were administered during TNBS and DSS-induced colitis revealed beneficial effects of SOD with regards to disease severity and molecular damage [59–62]. In studies of human colitis, MnSOD was strongly up-regulated in inflamed epithelium, whereas the Cu/ZnSOD content decreased with inflammation. On a cellular level, Cu/Zn and MnSOD were prominently present in neutrophils and macrophages, while extracellular (EC)-SOD was mainly localized in small vessels, stromal cells, and neutrophils. This differential regulation of the SOD isoforms indicates that ROS generation and its detoxification are important factors in the etiology of IBD, whose specific role needs to be clarified in future studies [63].

2.3 Nitric oxide synthases and the generation of reactive nitrogen species (RNS)

Nitric oxide (NO) is the key molecule during the generation of RNS (Figure 2). Since NO has a low molecular weight, is uncharged, and is soluble in both aqueous and hydrophobic environments, it is highly diffusible across cell membranes and through tissues. The diffusion coefficient for NO is 3300 μm²s⁻¹, which is comparable to that for O₂ (2800 μm²s⁻¹) and implies that NO is able to diffuse as far as 5–10 cell diameters in 1 sec [56]. NO production in cell culture studies coincides with the appearance of NO₂⁻ and NO₃⁻, which are formed at approximately equal and constant rates [64]. These metabolites arise from NO auto-oxidation by reacting with O₂ to form nitrogen dioxide radical (NO₂[•]) that can react further with NO₂[•] or NO to form N₂O₄ or N₂O₃, respectively. These higher N-oxides can subsequently form NO₂⁻, NO₃⁻ and nitroso products, which are closely related with enzymatic as well as non-enzymatic conversions with complex (patho-)physiological consequences [65–67]. Furthermore, NO reacts with O₂^{•-} at diffusion controlled rates to form peroxynitrite (ONOO⁻) [68] (Figure 2). ONOO⁻ represents an important source of a complex spectrum of highly reactive secondary radicals [42]. Its protonated form, ONOOH,

undergoes rapid ($t_{1/2} \sim 1$ sec) homolysis to form the strongly oxidizing $\cdot\text{OH}$ and the weaker oxidant $\text{NO}_2\cdot$. In addition, ONOO^- reacts with CO_2 , which is present in millimolar concentrations in tissues, to form nitrosoperoxycarbonate (ONOOCO_2^-), which decomposes with a half life of ~ 50 msec into $\text{NO}_2\cdot$ and highly reactive carbonate radical anions ($\text{CO}_3^{\cdot-}$) [69]. When the concentration of $\text{NO}\cdot$ exceeds that of $\text{O}_2^{\cdot-}$, which predominantly occurs within cellular membranes, autoxidation leads to the formation of $\text{NO}_2\cdot$ and N_2O_3 . N_2O_3 itself is an S- and N-nitrosating species, but readily decomposes with a half-life of 0.2 msec [56,69].

Three isoforms of NO synthases exist that can generate NO over a wide range of concentrations in an NADPH and O_2 -dependent process by converting L-arginine to citrulline and NO [70]. Neuronal and endothelial NOS produce NO at low levels in nerve and endothelial cells, respectively, while activation of inducible NOS (iNOS) leads to high NO concentrations in macrophages during inflammation (reviewed in [71]). In general, the biology of NO can be divided into two categories: (i) direct effects of NO mediated by chemical reactions that occur fast enough to allow NO to react directly with biological target molecules, and (ii) indirect effects that require that NO reacts with other ROS to generate RNS, which then react with biological targets [56]. Biological functions of NO are highly concentration dependent, with relevant concentrations varying over three orders-of-magnitude. One of the major challenges in the study of colitis has been to define the concentrations of NO arising in the inflamed colon and the biological consequences of the different concentrations of NO and its derivatives in the inflamed tissue. In this regard, significant insights have been obtained from computational modeling. By describing rates of reaction and diffusion in realistic geometries, mathematical modeling has estimated that maximal NO concentrations reached at the base of a crypt are in the sub-micromolar range (~ 0.3 μM) and the cumulative NO dose an epithelial cell is experiencing during migration from the base to the top of the crypt is ~ 560 $\mu\text{M min}$ [72] (Figure 3). Taking into account findings that epithelial cells contribute to the NO production in the colon [32,73,74] and that macrophages are able to infiltrate into the *lamina propria*, these models predict maximal NO concentrations of ~ 0.4 μM and maximal cumulative doses of ~ 1 $\mu\text{M min}$ [72] (Figure 3). These calculations are in accordance with cell culture studies demonstrating that upon stimulation macrophages produce levels of NO up to 1 μM [64]. As a rule of thumb, at low (nM) concentrations NO promotes cell survival and proliferation and acts as an important signaling molecule in the cardiovascular, nervous and immune systems, while, at higher concentrations (> 1 μM), such as may occur at sites of inflammation, it can be cytotoxic and cytostatic, inducing cell cycle arrest, apoptosis and senescence [56,69,75,76].

During colitis, iNOS is considered the main source of NO. In healthy mice, iNOS mRNA is expressed in the ileum, but not in the jejunum or colon. iNOS protein is also detected in normal ileal epithelial cells, suggesting that it may also fulfill a role in maintaining intestinal homeostasis [33]. In the inflamed gut, iNOS is present throughout the intestinal tract, including the jejunum and colon [26,27,33,37,77,78]. Induction of iNOS is regulated by neutrophils and macrophages in combination with cytokine stimuli, as suggested by the fact that depletion of Gr-1^+ neutrophils, clodronate-induced depletion of macrophages, and blocking of TNF- α in the colon, respectively, prevented iNOS induction [26,41]. Various

mouse studies demonstrated that colitis-induced activation of iNOS is accompanied by increased levels of NO-derived species in plasma and urine [26,38,39,79]. Saijo *et al.* studied the dynamics of biomarkers of NO production, such as NO_2^- , NO_3^- , and nitroso and nitrosyl species during DSS-induced chronic and acute colitis [80], and showed that blood levels of nitroso products correlated well with disease development. This correlation was also true for plasma levels of NO_2^- and NO_3^- , but only in acute colitis during the onset of the inflammatory response. A more complex response of these ions was observed in tissues as well as in the chronic colitis model. Interestingly, for all markers analyzed, similar changes were observed in organs other than colon, such as liver, brain, aorta, and heart, highlighting the systemic effects of this disease. In general, the study by Saijo *et al.* suggests that NO_2^- serves as a suitable indicator of colitis per se, although the rise of plasma NO_2^- may not correlate well with the progression and resolution of inflammation [80].

Supporting the notion that NO generation during colitis can be attributed to the activation of iNOS, no increase in NO-derived species was observed during colitis in *Il-10^{-/-}* mice in an *iNos^{-/-}* background [38]. Similarly, in *H. hepaticus*-infected *Rag2^{-/-}* mice concurrent administration of an iNOS inhibitor prevented NO_3^- production and reduced epithelial pathology and cancer incidence [26]. Similarly, an iNOS inhibitor significantly reduced colonic adenocarcinoma formation in a dose-dependent manner in a CAC model using DSS-treated *Apc^{Min/+}* mice [81]. These effects can be directly attributed to iNOS inhibition, since during *H. hepaticus*-induced colitis, iNOS inhibitor treatment had no influence on the numbers of neutrophils and macrophages [26]. Interestingly, administration of iNOS inhibitors also decreased the levels of pro-inflammatory cytokines such as TNF- α and IL-1 β in DSS-induced colitis, indicating a positive-feedback regulation of these components under inflammatory conditions [79,81]. The notion that ONOO⁻ can not only act as a downstream effector molecule, but also as an active driving force of disease development is supported by the finding that intrarectal administration of ONOO⁻ itself was sufficient to induce the generation of NO-derived species, iNOS expression, and colitis in rats [82].

Ambiguous results were obtained from genetic studies of colitis in *iNos^{-/-}* mice. Several groups reported a protective effect of iNOS depletion on tissue inflammation, damage, and cancer rates [39,78,83–85], whereas others found no effect [38,86] or even more severe tissue damage and pathology in *iNos^{-/-}* mice [37,87]. Krieglstein *et al.* performed bone marrow transplantation experiments using iNOS-proficient or -deficient cells transplanted into wild-type or *iNos^{-/-}* mice. In mice deficient in either tissue or blood cell iNOS, or both, DSS-induced colitis was attenuated. Interestingly, MPO activity was also lower in *iNos^{-/-}* mice and occurred at the lowest levels in *iNos^{-/-}* mice transplanted with blood cells carrying functional iNOS. These results suggest that both blood-cell and tissue-derived iNOS plays a role in DSS-induced colitis, with tissue-associated iNOS making a larger contribution to the recruitment of MPO-positive inflammatory cells [84]. Similarly, Beck *et al.* showed that chimeric mouse lines with iNOS-deficient blood cells were more resistant to DSS and TNBS-induced colitis; in the DSS model neutrophils were the main source of iNOS [88]. This is in agreement with our finding that depletion of Gr-1⁺ neutrophils of *H. hepaticus*-infected mice prevented iNOS induction in the colon [26]. Another study analyzed mouse lines deficient in iNOS, nNOS, eNOS or e/nNOS and revealed that loss of

iNOS or eNOS was protective on DSS-induced colitis, whereas loss of nNOS resulted in more severe disease and higher mortality rates. These results indicate that NO produced by individual isoforms plays different roles in the modulation of intestinal inflammation [85]. In terms of signature biomarkers of iNOS activity, *H. hepaticus*-induced colitis in *Rag2*^{-/-} mice led to significantly higher levels of nitrotyrosine in colon tissue as well as in serum [29]. Similarly, TNBS-induced colitis led to increased levels of nitrotyrosine [39]. In this model, ablation of iNOS resulted in reduced levels of nitrotyrosine indicating that iNOS is required for the generation of nitrosative damage [39]. On the other hand, in *iNos*^{+/+} and *iNos*^{-/-} mice with chronic DSS-induced colitis showed no differences in nitrotyrosine levels, pointing to a role of MPO activity (see below) [86]. These apparently contradictory results may be reconciled by a mechanism in which depletion of iNOS has a protective effect mostly in models of acute, severe colitis, whereas, in models of chronic colitis, depletion of iNOS has either no effect or results in more severe pathology [37,38,86,87]. The different effects of iNOS depletion in different colitis models may therefore be dependent upon the severity of disease at the time point analyzed and may represent the double-edged role of NO as a signaling molecule on the one hand and a tissue-damaging factor on the other. Moreover, there is evidence for endogenous production of NO by iNOS in epithelial cells [87,88[73,74]. Recently, Shaked *et al.* demonstrated that mice with a deletion in one allele of the *Apc* gene specifically in intestinal epithelial cells (IEC) in combination with constitutively active NF-κB signaling in these cells (*Ikkβ(EE)*^{IEC}/*Apc*^{+/-} IEC mice) show increased levels of iNOS expression, DNA damage induction and elevated colon carcinogenesis [32]. Interestingly, these mice developed intestinal tumors despite the absence of signs of pathology and hyperplasia typical of colonic inflammation. Treatment of these mice with an iNOS inhibitor reduced cancer rates and molecular damage markers highlighting the iNOS-dependency of the observed effects (see below for further discussion of this mouse model) [32].

Although there is a controversy about the factors that trigger human macrophages to produce NO and the quantity of NO generated [89–92], the results obtained with rodent models of IBD have largely been confirmed in humans. Thus, increased iNOS expression and activity were also identified in patients with Crohn's disease and ulcerative colitis [73,74,93,94]. Interestingly, as observed in mouse models, iNOS and nitrotyrosine were not only detected in mononuclear cells, but also in neutrophils, which suggests a significant role for this cell type in NO production during human disease [74]. Several studies have found a correlation between increased iNOS expression during IBD and nitrotyrosine levels and NO production itself [73,74,94–97], but correlation of NOS activity with severity of disease was not always evident, and profound differences in molecular fingerprints existing between Crohn's disease and ulcerative colitis [93,98,99]. In terms of biomarker development, a recent study of 38 patients with ulcerative colitis and 42 patients with Crohn's disease revealed only slightly elevated plasma levels of nitrotyrosine in active compared to inactive IBD, and no significant differences were found compared to non-IBD control subjects. These results suggest that NO-derived damage is less pronounced in humans compared to rodent colitis models and that nitrotyrosine may not be the most suitable biomarker of human disease [29].

2.4 MPO and the generation of HOCl

Hypochlorous acid (HOCl) is one of the most potent antimicrobial compounds released by activated immune cells as a first-line defense mechanism against invading pathogens. During inflammation, HOCl could lead to injurious reactions in surrounding host tissue. HOCl is generated from Cl^- and H_2O_2 by heme peroxidases, such as neutrophil MPO or eosinophil peroxidase (EPO) (Figure 2). Analogous reactions generate hypobromous acid (HOBr) and hypothiocyanous acids (HOSCN). All of these molecules are particularly reactive with thiols, methionine, and tyrosine, and are also capable of damaging nucleobases (see below, reviewed in [42,100,101]). For a more in-depth discussion of the various and complex reactions mediated by MPO, the reader is referred to recent reviews [102,103].

Although there are reports that MPO is expressed in specific monocytes and macrophages, its major site of expression is the neutrophil, and MPO immunostaining is often used as a marker for the *in situ* detection of neutrophils. Within neutrophils, MPO is stored mainly in azurophilic granules, which are released into the extracellular space during neutrophil activation [103]. It is therefore not surprising that MPO protein levels and activity are elevated during colitis, since neutrophils are recruited to sites of inflammation [27,79,104]. In mouse models of *C. rodentium*-induced acute colitis and *H. hepaticus*-induced chronic colitis, infiltration of MPO-positive cells was associated with increased tissue and serum levels of protein damage markers of HOCl (e.g., chlorotyrosine) [27,29,104]. Interestingly, in both mouse models, increases in chlorotyrosine were much more pronounced than increases in nitrotyrosine, indicating that MPO-mediated halogenation chemistry is of central importance during infection-induced colitis in mice. Increases in nitrotyrosine in these mouse models may be also attributable to MPO activity, since it was shown that MPO (and EPO) can use H_2O_2 to convert NO_2^- to NO_2^\bullet via a nitryl chloride (NO_2Cl) intermediate [105]. During inflammation this process may also contribute to cytotoxicity mediated by tyrosine nitration and chlorination, and other macromolecular damage. Using MPO- and EPO-deficient mice, Brennan *et al.* demonstrated that MPO and EPO participate in nitrotyrosine formation *in vivo*, possibly by generation of NO_2^\bullet and, to a lesser extent, ONOO^- ; in some instances, MPO and EPO accounted for the majority of the nitrotyrosine formed [106]. Similar results were obtained in a model in which peritonitis was induced either by cecal ligation and puncture or by infection with *K. pneumoniae*; only the latter led to an increase of total NO_2^- and NO_3^- levels of about 20-fold and to increased levels of nitrotyrosine [107]. In contrast, nitrotyrosine levels remained unchanged in *K. pneumoniae*-infected mice deficient in MPO, supporting the view that MPO is capable of generating RNS *in vivo* [107]. Also noteworthy was the finding in a human study that increases in nitrotyrosine in ulcerative colitis correlated only with MPO expression and not with that of iNOS [53].

In contrast to reports of studies in *iNos*^{-/-} mice, none have been reported in *Mpo*^{-/-} - deficient mice to assess the role of MPO during colitis and CAC. However, some information is available from other pathological states. While *Mpo*^{-/-} mice were more susceptible to several types of infections, such as infections with *Candida albicans* and *Klebsiella pneumoniae*, they paradoxically showed more severe atherosclerosis [108]. Additionally, deficiency in MPO was associated with increased infarct volumes and

nitrotyrosine formation in a mouse model of ischemic brain injury [109]. These studies thus suggest a protective, anti-inflammatory role of MPO, in at least select inflammation-related pathologies [103,108,109]. Thus, the possibility cannot be excluded that MPO expression and activity during colitis might serve as in part a mechanism of tissue protection rather than tissue destruction. Studies in *Mpo*^{-/-} mice with respect to colitis and colon cancer development would help to clarify this interesting question.

In the context of the fact that MPO expression is well correlated with human IBD, a recent study revealed that MPO immunostaining may serve as marker to assess disease activity in patients with ulcerative colitis [110]. This is consistent with our own study in patients with ulcerative colitis and Crohn's disease where MPO-derived chlorotyrosine was significantly elevated in serum from patients with ulcerative colitis and Crohn's disease, potentially contributing to a set of biomarkers for IBD activity in human serum [29].

2.5 Antioxidant defense

Efficient antioxidant defense systems exist to keep the cellular redox state in balance. The most prominent ones include the glutathione/glutathione disulfide (GSH/GSSG), cysteine/cystine (Cys/CySS), and thioredoxin/thioredoxin disulfide (TRx/TRxSS) redox couples. These systems maintain mucosal integrity and intestinal homeostasis, which is evident by the fact that, e.g., GSH is present in millimolar concentrations in the intestinal epithelium and its *de novo* synthesis can be efficiently induced upon pathological stimuli. Nevertheless, conditions of oxidative and nitrosative stress can occur if ROS and RNS levels exceed the cellular antioxidative capacity.

Evidence supporting an active role of antioxidant defense mechanisms in the control of intestinal homeostasis comes from both mouse and human studies. Ardite *et al.* suggested that TNBS-induced colitis is triggered by ROS generation followed by GSH depletion. Following treatment with TNBS, GSH pools in colon mucosa were found to be depleted, an effect that was inhibited by administration of the GSH precursor and antioxidant *N*-acetylcysteine (NAC), which resulted in significantly less histological injury. Underpinning the dynamic and tightly regulated control of antioxidant systems, GSH levels recovered after ~1 week of TNBS treatment due to induction of γ -glutamylcysteine synthase gene expression [111]. NAC-protective effects were also observed in a long-term DSS-induced colitis model, in which NAC treatment reduced histopathology accompanied by reduced numbers of nitrotyrosine and iNOS-containing cells in the mucosa. Mechanistically, the finding that NAC administration reduced tumor incidence may be attributed to sensitization of epithelial cells to apoptosis [112]. Redox regulation also plays an important role in human IBD, which is evident by findings that intestinal subepithelial myofibroblasts in patients with Crohn's disease exhibited an increased oxidative state due to a decrease in the GSH/GSSG ratio [113] and GSH content in tissue from colorectal tumors was much lower than in non-malignant tissue [114].

Several enzymes are involved in the intra-intestinal antioxidant defense. For example, glutaredoxins (GRx) and glutathione reductase (GSR) catalyze the reduction of GSSG through thiol-disulfide exchanges. Similarly, thioredoxin reductases (TxnRD) are involved in the regeneration of thioredoxin. Glutathione peroxidases (GPx) are the major H₂O₂-

reducing intestinal selenoproteins and GSH S-transferases catalyze GSH-detoxification reactions of luminal electrophiles and carcinogens (reviewed in [115]). These antioxidant defense systems are dynamically regulated in the course of colitis development as recently revealed by sensitive qPCR-array-based gene expression profiling in *H. hepaticus*-infected *Rag2*^{-/-} mice [27]. Unexpectedly, several antioxidant enzymes, such as MnSOD and catalase, showed modestly decreased levels in inflamed tissue, whereas others, including glutathione and thioredoxin reductases (*Gsr* and *Txnrd1*) as well as *Gpx2*, displayed higher expression levels, which points to complex regulatory networks that counteract oxidative stress during colitis.

In particular, GPx2 is an interesting factor in the etiology of colitis. GPx2 is a gastrointestinal-specific form of GPx that is highly expressed at crypt bases. Similar to other glutathione peroxidases, GPx2 reduces hydroperoxides using GSH. However, GPx2 seems to play a double-edged role during the etiology of IBD [116]. GPx2 is up regulated during colitis, and possibly could protect colon tissue by reducing concentrations of inflammation-derived hydroperoxides, which may otherwise give rise to highly reactive •OH [117]. In accordance with this view, *Gpx2*^{-/-} mice were susceptible to DSS-induced colitis and *Gpx2*^{-/-} mice treated with a combination of DSS and azoxymethane (AOM) tended to develop higher incidence of tumors than wild-type animals [118]. Of note, tumor incidence in this model showed a strong positive correlation with inflammation scores [116]. Some defects caused by GPx2 deficiency are thought to be compensated by up-regulation of GPx1 [119]. This becomes more evident by the finding that in GPx1/2 double deficiency spontaneous development of colitis was accompanied by elevated levels of MPO activity, increased mutation frequencies, and spontaneous cancer development [120]. In spite of the evidence for a protective role, tumor sizes were smaller in *Gpx2*^{-/-} mice. This has been associated with anti-apoptotic and pro-proliferative properties of GPx2 and highlights the double-edged role of this enzyme in CAC [116,118,119]. This view is consistent with human data showing that expression levels of GPx1 and GPx3 were significantly decreased in colorectal tumor samples, whereas those of GPx2 were increased [114].

Gpx2 expression is regulated by the transcription factor NRF2, which acts as a cellular master switch in controlling expression of a plethora of oxidative stress response genes. Its activity is controlled by KEAP1, which binds to NRF2 and retains it in the cytosol, inducing its degradation via ubiquitylation. Upon oxidation or nitrosylation of specific thiol residues, KEAP1 releases NRF2 to the nucleus, where it induces expression of antioxidant and detoxifying genes, such as heme oxygenase, GSTs, and GPx2 [121]. It is important to note that in addition to their direct role in redox regulation, GSH and TRx serve as trans-nitrosylation agents with functional consequences on several important biochemical signaling pathways [122]. Because GSH is the predominant pool of thiol groups in cells, it can serve as the initial pool of nitrosothiols (RSNO), which are then available for transnitrosylation to many other proteins and peptides [123]. For example, it was shown that GSNO is able to site-specifically transnitrosate TRx in a redox-controlled manner which is then able to catalyze caspase-3 nitrosation leading to inhibition of cell death [124–126]. With regard to NRF2 regulation, studies in the human colon cancer cell line HCT116 revealed that S-nitrosylation can have direct functional consequences to cellular mechanisms

in response to oxidative stress by regulating NRF2 activity [34]. Thus, direct S-nitrosylation of KEAP1 may trigger nuclear translocation of NRF2, where it induces protective responses to counteract NO-induced damage to prevent CAC. (*N.B.*: As discussed below, this may contribute to the relative resistance of HCT116 cells to NO-induced cytotoxicity [34]). Support for such a hypothesis comes from several animal studies demonstrating that *Nrf2*^{-/-} mice have an increased susceptibility to DSS-induced colitis and associated colon cancer. Specifically, increases in the severity of colitis and cancer incidence in *Nrf2*^{-/-} mice were associated with repression of antioxidant/detoxifying genes and increased expression of mediators of inflammation such as iNOS, MPO, IL-1 β , and TNF α as well as increased levels of nitrotyrosine staining [127–129]. Interestingly, Choi *et al.* showed that *Nrf2* expression is modestly suppressed by oxidative stress during TNBS-induced colitis. NRF2 stimulation by sulforaphane significantly reduced DNA instability during inflammation, indicating that suppression of NRF2 by unknown mechanisms has functional consequences in CAC [57]. The finding that *Nrf2* is suppressed during colitis may also account for the down-regulation of some antioxidant and detoxifying genes in *H. hepaticus*-induced murine colitis [27]. Significantly, an epidemiological study suggested that a promoter polymorphism in the human *Nrf2* gene is associated with ulcerative colitis in a Japanese population [130].

In summary, these results provide a potential foundation for development of pharmacological intervention to treat IBD. Several mouse studies with antioxidant compounds have shown promising results, while clinical trials with these compounds largely proved to be negative because of adverse effects, non-specific drug distribution, and low retention in the colon [131–133]. A recent report addressed the pharmacokinetic and pharmacodynamic problems encountered in previous studies by using NO-containing nanoparticles that exhibit antioxidant properties. These particles showed improved accumulation and retention in the colonic mucosa and less up-take into the blood circulation compared to the low-molecular weight analogue TEMPOL. Subsequently, therapeutic effects were significantly improved in a mouse model of DSS-induced colitis, suggesting that this type of treatment could offer a useful perspective for human therapy [134].

3 Genotoxicology of ROS and RNS during inflammatory bowel disease

Many of the chemical mediators of inflammation are thought to act as anti-microbial factors or signaling molecules, depending on their source and local concentration. In particular oxidative and nitrative modification of proteins and changes in the cellular oxidative state are thought to fulfill various signaling functions that lead to diverse biological outcomes, such as cell proliferation, cell cycle regulation, and induction of senescence and apoptosis. On the other hand, these reactive chemical species can damage all classes of cellular biomolecules, including DNA, by both direct and indirect mechanisms, which elicit mutagenic and cytotoxic effects. In the following section, we provide an overview of the genetic toxicology of inflammation-related chemical species during colitis. We begin with an overview on RNS-related cytotoxicity studies, and then examine more closely genotoxicological mechanisms involved in murine colitis and human IBD. Finally, we discuss the regulation of DNA repair mechanisms during colitis and summarize work providing evidence that non-repaired, persistent damage products contribute to genomic mutations that initiate and promote CAC.

Cytotoxicity Application of precise and biologically relevant concentrations of NO and its derivatives is essential to mimic the biological environment and to study the physiological and cytotoxic functions of these chemical species. In general, NO can be delivered to cells via three approaches: NO donor compounds, such as NONOates, NO gas, or NO produced by activated macrophages [135] (Figure 4 A–C). The most rigorous conditions are achieved by using of NO and O₂-permeable polydimethylsiloxane (Silastic) tubing to deliver gases at constant and predictable rates. These delivery systems are specifically designed and optimized to provide controlled, steady state concentrations of NO, O₂, and NO₂, thereby mimicking the chemical environment thought to exist in inflamed tissues [136–140]. The range of estimated concentrations of NO in inflamed tissue, as obtained by mathematical modeling (Figure 3), are in reasonable agreement with those found to be cytotoxic in studies applying NO and O₂ via Silastic tubing to cell culture media (Figure 4 D). For example, the minimum NO dose that was associated with cytotoxicity in TK6 human lymphoblastoid cells was ~150 μM min [141]. Interestingly, TK6-isogenic cells that lack p53 (NH32 cells) [141] and cells derived from colonic origin (HCT116 cells) [34,139] were more resistant, displaying threshold doses of 300 μM min and 1000–1600 μM min, respectively (Figure 4 D). These findings may be related to the importance of p53-dependent apoptosis in cytotoxic mechanisms (see below) and to the activation of the NRF2-KEAP1 signaling pathway to mitigate NO-mediated damage (see above), respectively. NO production in macrophages stimulated by LPS and IFN-γ resulting in growth inhibition and impaired cell viability, which could be attenuated by addition of the NOS inhibitor, NMA [142]. Moreover, co-culture of TK6 cells with stimulated macrophages also resulted in a substantial decrease in cell viability, both in the macrophages and the TK6 cells. Again, application of NMA reduced cytotoxicity in both cell types, which verifies the role of NO in the induction of these responses [143].

These results provide strong evidence that local NO concentrations, as they exist in the vicinity of activated immune cells, can reach pathophysiological and cytotoxic levels *in vivo*. Mechanistic studies revealed that NO-mediated cytotoxicity is an extremely complex process involving several cell death pathways, including apoptosis and necrosis, depending on the cell type studied. Potential mechanisms include energy depletion upon acotinase inhibition, lipid peroxidation and protein modification through nitrotyrosine formation [144]. However, the earliest cellular effects observed include the inhibition of DNA synthesis, most likely as a result of DNA damage, leading to cell cycle arrest and apoptosis induction [145]. Most of the cells, however, will experience sub-toxic doses of ROS and RNS, causing macromolecular damage. As discussed in the following sections if such damage occurs in DNA, this may lead to mutational events driving tumor initiation and progression.

3.1 DNA damage during inflammatory bowel disease

ROS and RNS-mediated DNA damage products span a wide range of reactions, including nitrosation, nitration, oxidation, and halogenation reactions (Figure 5), which are discussed in the following with a special emphasis on studies analyzing murine and human colitis. For a more general overview of the chemical biology of oxidative and nitrosative DNA damage the reader is referred to more specific reviews on this topic [146,147].

3.1.1 Nitrosative deamination— N_2O_3 which is thought to be the primary agent causing nitrosative deamination of DNA. Other mechanisms include spontaneous hydrolysis and enzymatic processes by deaminases. DNA damage products formed by nitrosative deamination include 2'-deoxy-uridine (dU) derived from cytosine, 2'-deoxy-inosine (dI) derived from adenine, 2'-deoxy-xanthosine (dX) or 2'-deoxyoxanosine (dO) derived from guanine, and 2'-deoxythymidine (dT) derived from methylcytosine [69,148] (Figure 5). Due to their mutagenic potential, formation of these damage products is of high functional relevance. For example, during replication formation of deamination products mainly cause single-base transition mutations such as A:T to G:C (dI), C:G to T:A (dU), and G:C to A:T transitions (dX). In addition to deamination of exocyclic amines, nitrosation of heterocyclic nitrogens such as N^7 or N^3 in dA and N^7 in dG can induce depurination creating abasic sites [149]. In an attempt to define the spectrum of nitrosative DNA lesions induced by RNS, purified DNA was exposed to NO (1.3 μ M) and O_2 (190 μ M) [149]. Subsequent quantification of several DNA adducts by stable isotope-dilution mass spectrometry revealed that dX, dI and dU were formed at nearly identical rates to the extent of ~ 80 lesions per 10^6 nt after 12 h exposure to NO. Furthermore, abasic sites were generated at a rate of ~ 10 per 10^6 nt after 12 h of exposure to NO. In combination with other studies, which demonstrated that nitrosative guanine deamination can lead to the formation of interstrand cross-links (see below) [150], it can be estimated that under these conditions the following spectrum of nitrosative DNA damage occurs: 25–35% each of dX, dI, and dU, 4–6% abasic sites and $\sim 2\%$ dG-dG cross-links [149]. In cell culture, a ~ 4 -fold protective effect by the cellular environment was observed against the formation of dX, dI and dU. Notably, significant elevations of these products of ~ 4 -fold were only observed when cells were exposed to cytotoxic doses of NO and N_2O_3 (*i.e.*, 1.75 μ M NO and 186 μ M O_2 for >12 h) [135,151]. An earlier study detected a similar increase in dX levels of about ~ 6 -fold using stimulated macrophages [152]. The slightly higher values obtained in the latter study may be related to the possibility that intracellular NO levels in stimulated macrophages as used in the study by Dong and Dedon may exceed local concentrations of 1–2 μ M [135,152]. *In vivo*, recent results obtained from *H. hepaticus*-infected *Rag2*^{-/-} mice revealed a small increase in levels of dI in inflamed colon tissue by 26%, whereas similar levels of dX were observed in colitis-affected and control tissue [27].

Taken together, these results indicate that nitrosative deamination products arise in cells and tissues under conditions of inflammation, but the relatively small increases suggest either limited nitrosative chemistry in nuclear DNA or effective protection and repair mechanisms, both in cell culture models and in the mouse [27,135]. That this holds true for the human situation awaits further study.

3.1.2 DNA oxidation—DNA can also be directly damaged by nucleobase oxidation by ONOO⁻ and ONOOCO₂⁻ derived oxidants such as OH[•], NO₂[•], and CO₃^{•-} [148]. While ONOO⁻ itself, mainly causes 2-deoxyribose oxidation (*i.e.*, strand breaks) and some base oxidation, ONOOCO₂⁻ causes mainly base oxidation [153]. Due to its low reduction potential, guanine represents the most vulnerable nucleobase, which shows DNA sequence- and agent-specific variations in damage chemistry [147,154–156]. Consequently, *in vitro* experiments demonstrated significant reactivity of ONOO⁻ only with dG, leading to the

formation of two major oxidation products, *i.e.*, 8-oxo-7,8-dihydro-2'-deoxy-guanosine (8-oxo-dG) and 8-nitro-dG [147,157] (Figure 5). Apart from iNOS- and NADPH oxidase-dependent generation of ROS and RNS, which is thought to occur mainly in macrophages, nitration and oxidation of dG can also be induced by neutrophils via MPO-dependent reactions that generate NO_2^{\bullet} [158,159]. 8-oxo-dG and 8-nitro-dG are significantly more reactive with ONOO^- than dG itself, however 8-nitro-dG is not stable and depurinates to 8-nitro-G generating an abasic site in the DNA backbone ($t_{1/2} \sim 1$ h at 37°C), which can cause base substitutions such as G \rightarrow T transversions [147,157]. In contrast, 8-oxo-dG is more stable than 8-nitro-dG, but exhibits a $\sim 1,000$ -fold higher reactivity than dG and gives rise to more than 10 different secondary oxidation products [153]. It is important nonetheless to keep in mind that even a 1000-fold difference in the reactivity of dG and 8-oxo-dG means that dG is still the major target in cellular DNA when 8-oxo-dG is present at steady state levels of 1–10 per 10^6 nucleotides [151,154]. Secondary dG oxidation products include spiroiminodiydantoin (Sp), guanidinohydantoin (Gh), and oxalzone (Ox), all of which have been extensively described *in vitro* [154,160–163]. In contrast to nitrosative DNA damage that mainly causes transition mutations, oxidative lesions tend to cause transversion mutations. While 8-oxo-dG itself can lead to C:G to A:T transversion mutations [164], secondary oxidation products such as Sp cause G:C to T:A and G:C to C:G transversions and can act as a strong inhibitor of DNA polymerase extension leading to replicative stress. Similarly, Gh lesions are also highly mutagenic causing G:C to C:G transversions, though they can be bypassed nearly as efficiently as 8-oxo-dG. For both secondary damage products, mutation frequencies are at least an order of magnitude higher than for 8-oxo-dG [165].

Because of their promutagenic potential, significant biological consequences are expected if such primary and secondary oxidation products are formed in mammalian DNA *in vivo*. While 8-oxo-dG is one of the most widely studied DNA damage lesions *in vivo* [166], data on the existence of guanine secondary oxidation products is not as abundant. Our recent LC-MS/MS-based study on a comprehensive set of inflammation-related DNA damage products in *H. hepaticus*-infected *Rag2*^{-/-} mice aimed to detect guanine secondary oxidation products in mammalian tissues. In fact, Sp and Gh were detected in mouse liver and colon at absolute levels of ~ 1 – 5 lesions per 10^8 nucleotides, which are ~ 100 times lower than those of 8-oxo-dG. Interestingly, levels of Sp and Gh displayed tissue-specific differences and correlated strongly with their parent molecule 8-oxo-dG, indicating that these molecules indeed arise from 8-oxo-dG *in vivo*. In this study, no strong influence of colitis induction on whole tissue levels of 8-oxo-dG and its secondary oxidation products was observed. Thus, while 8-oxo-dG levels showed a slight and transient decrease during colitis development, levels of Sp tended to correlate positively with corresponding colonic histopathology indices, suggesting that these lesions may have biological significance during inflammation [27]. Similarly, Shaked *et al.* found modestly elevated levels of oxidative DNA damage lesions, such as 8,5'-cyclo-2'-deoxyadenosine (cdA) and 8,5'-cyclo-2'-deoxyguanosine (cdG), in intestinal epithelium in a mouse model with constitutive induction of iNOS expression measured by LC-MS/MS analysis (see above) [32]. It is important to stress that these studies analyzed lesion frequencies in tissue samples. Therefore, even small changes as detected in these studies may be related to more drastic effects on a single cell level.

Although immuno-chemical methods of DNA damage detection based on DNA-damage-specific antibody binding have their limitations such as lack of chemical specificity and high background signals, these methods allow assessment of DNA damage on a single cell level. In this respect an analysis of an immunological colitis model detected elevated immuno-reactivity towards 8-oxo-dG and 8-nitro-G in nuclei of epithelial cells. These levels correlated with iNOS expression, suggesting a functional interplay between those factors [167]. Furthermore, stronger 8-oxo-dG immuno-reactivity was also detected in colonic epithelia of DSS-treated mice - an effect that could be almost completely suppressed by ectopic expression of extracellular SOD, which also considerably improved disease severity [61]. Interestingly, systemic effects of oxidative DNA damage were identified in mouse models of DSS-induced and immune mediated colitis using *Il-10*^{-/-} mice: circulating blood leukocytes isolated from diseased mice showed significantly increased 8-oxo-dG immuno-reactivity, which correlated to oxidative damage detected in inflamed tissue [168]. Such systemic effects may be related to activation of circulating innate immune cells and other factors or through the effect of circulating cytokines and acute phase proteins that autonomously induce oxidative stress in circulating peripheral cells [168].

There is also considerable evidence for the formation of oxidative damage during human IBD. For example, using the reduced-thiol-specific probe ¹⁴C-iodoacetamide (IAM), McKenzie *et al.* examined the thiol redox status as an indicator of oxidative protein damage and detected increased levels of oxidated thiol groups in colon epithelial crypt cells from IBD patients [169]. Furthermore, higher levels of 8-oxo-dG were detected in colorectal tumor tissue [114] and in inflamed tissues and tumors from patients with ulcerative colitis [170]. However, results from the latter study must be interpreted with caution, since it reported absolute values of >1 lesion/10⁴ bp, which is at least two orders of magnitude higher than widely established basal levels and raises the possibility of adventitious oxidation during sample manipulation [170]. Interestingly, genomic hot-spots of oxidative DNA damage seem to exist, which is evident by the findings that p53 and TGF- β genes in *H. pylori*-infected individuals showed higher levels of oxidative damage than BAX and β -ACTIN, as analyzed by an OGG1-based PCR method [171]. The basis for this observation awaits further clarification, but may include sequence-specific events, the transcriptional or replicative state of the gene locus, the nature of the damaging agent, or the type of DNA lesion [171].

In summary, a large body of evidence demonstrates that oxidative damage to DNA plays an important role during CAC, but so far the exact mechanisms are poorly understood. Another level of complexity is added by the recent finding that oxidatively-induced DNA damage may also interfere with epigenetic mechanisms that contribute to CAC. In this respect, O'Hagan *et al.* reported that, in cells treated with H₂O₂ and in a mouse model of acute colitis, oxidative stress causes enrichment of members of the silencing complex including DNMT1 at promoter CpG islands, which may be related to a role of DNMT1 in DNA repair. This may result in epigenetic alterations in affected cells [172].

3.1.3 Lipid peroxidation-induced damage—Apart from direct oxidative damage, DNA nucleobases can be damaged by oxidation products arising from lipid peroxidation of polyunsaturated fatty acids. For example, peroxidation of linoleic acid gives rise to several

α,β -unsaturated aldehydes, such as trans-4-hydroxy-2-nonenal (4-HNE), acrolein, and malondialdehyde (MDA), which can react with nucleobases to form exocyclic etheno adducts, such as 1-N⁶-2'-deoxy-ethenoadenosine (edA) and 3-N⁴-2'-deoxy-ethenocytidine (3-N⁴-εdC), glyoxal adducts, and a series of other adducts [69,135] (Figure 5). Several repair mechanisms exist that act on lipid peroxidation products, including base excision repair, direct reversal by AlkB family proteins, nucleotide excision repair and homologous recombination (see below) [173]. If persistent, lipid peroxidation adducts exhibit strong promutagenic potential causing transversion and transition mutations, depending on the nature of the adduct. Additionally, lipid peroxidation adducts can interfere with DNA replication and gene transcription, thereby contributing to cytotoxicity.

Elevated levels of these adducts were identified in several inflammatory conditions. Strong evidence for sustained formation of lipid peroxidation adducts comes from the SJL mouse model of NO overproduction, in which RcsX-tumor bearing SJL mice develop severe inflammation in the spleen and liver. Increased levels of DNA etheno adducts were not only observed in liver and spleen, but also in kidney, which again points to systemic effects, since the kidney is not considered a primary target of inflammation in this model [174]. Increases in etheno adducts were also observed in DSS-induced colitis in mice [175] and in tissues from patients with Crohn's disease and ulcerative colitis [173,176–178]. Increased levels of 4-HNE and MDA have been detected in biopsies from colorectal cancers, and were associated with dysregulation of antioxidant factors such as GPx1–3, SOD1, and GSH. This illustrates the pleiotropic cellular consequences of conditions of oxidative stress [114]. In addition to iNOS-dependent initiation of lipid peroxidation, this process may be also driven by MPO, as evident from the finding that *Mpo*^{-/-} mice generated less lipid peroxidation intermediates compared to wild-type mice in a peritonitis model [179]. H₂O₂ may also play a significant role in the initiation of the lipid peroxidation cascade during colitis, since DSS-treated mice with ectopic expression of EC-SOD displayed lower levels of MDA compared to untreated controls as evaluated by immunohistochemical staining of mucosal epithelial cells [61].

Paradoxically, several studies reported decreased levels of lipid peroxidation products in the course of inflammatory diseases. For example, Godschalk *et al.* detected decreased levels of lipid peroxidation-induced DNA damage in the onset of atherogenesis in apolipoprotein E deficient mice [180]. These results are similar to those in *H. hepaticus*-induced colitis, which revealed transiently reduced levels of DNA-etheno adducts in the course of the disease. The finding that levels in RNA remained unaffected suggests that either DNA repair or protective effects of the nuclear environment underlie the transient decrease in etheno adducts, rather than changes in the cellular radical load. Inconsistent results were also obtained in human studies, in which lower levels of etheno adducts were detected in peripheral blood lymphocytes from patients with colorectal cancer. In this study, mRNA levels of some repair enzymes (*i.e.*, glycosylases and APE1) were higher in peripheral blood lymphocytes of colon cancer patients than those of controls, but did not lead to higher excision rates of etheno adducts. In contrast, in target colon tissues, excision rates of etheno-adducts were higher compared to control tissue, while levels of DNA etheno adducts were

lower in tumors than in non-tumorigenic tissue, pointing to complex local and time-dependent changes in adduct levels during disease development [181].

In summary, alterations in lipid peroxidation adducts have been reported in several mouse models of colitis as well as in human disease, however these changes exhibit a dynamic nature and are highly dependent on the model and condition analyzed. The molecular bases for these inconsistencies remain unclear, but may be related to dynamic regulation of the repair efficiencies of these adducts.

3.1.4 Halogenation-induced damage—The reaction of nucleobases with MPO-derived HOCl leads to the formation of products such as 5-chloro-2'-deoxy-cytidine (5-Cl-dC), 5-chloro-2'-deoxy-uridine (5-Cl-dU), as a deamination product of 5-Cl-dC, 8-chloro-2'-deoxy-guanine (8-Cl-dG), as well as dihalogenated nucleobases (Figure 5). All of these molecules have been detected in cellular RNA as well as in murine and human DNA under inflammatory conditions, such as sepsis and atherosclerosis, and were therefore proposed as potential biomarkers of inflammatory diseases [182–189]. Of note, following LPS treatment, 8-Cl-dG was only observed in the liver of wild-type but not of *Mpo*^{-/-} mice, which indicates that *in vivo* formation of halogenation damage predominantly depends on this enzyme [182]. Repair mechanisms for these adducts are poorly characterized, but potentially rely on base excision repair (BER), since free base adducts, such as 8-Cl-dG, were detected in urine of LPS-treated rats [182]. In particular, 5-Cl-dC appears to be of biological relevance, since it mimics 5-methylcytosine and thereby enhances binding of the binding domain of methyl-CpG-binding protein 2 (MeCP2) and serves as a substrate for DNMT1 [190,191]. It is plausible that, within a CpG sequence context, this results in heritable, reversible gene silencing, with altered cytosine methylation patterns providing a potential link between inflammation, epigenetic changes, and tumor initiation [192]. CpG hypermethylation has been associated with CAC revealing high methylation frequencies in several genes, including the tumor suppressor p16 (see below) [193]. Hypermethylation was also present in non-dysplastic, normal-appearing epithelium from patients with ulcerative colitis, but not from control individuals, indicating that methylation precedes dysplasia [193]. In this context, it is interesting to note that a polymorphism in the *MPO* gene (G463A) associated with reduced MPO mRNA expression reduces the risk for lung cancer development [194–196]. Regarding formation of chlorination adducts during murine colitis, our recent studies in *H. hepaticus*-infected *Rag2*^{-/-} mice demonstrated significantly elevated levels of adducts in DNA (5-Cl-dC) and RNA (5-Cl-rC) in colitis-affected mice [27]. While increases in DNA and RNA were modest (~25%), more drastic changes were observed on the protein level, with 3-Cl-Tyr levels below the limit of detection in control mice, but clearly detectable in all *H. hepaticus*-infected mice at 20 weeks post infection [29]. These results indicate that either proteins are more accessible to damage induction and/or damaged nucleic acids are less stable (RNA) or efficiently repaired (DNA). Nevertheless, it is important to note that mild increases in damage levels in whole colonic DNA may correspond to drastic changes on a single cell level, potentially contributing to cellular transformation and colon cancer development in *H. hepaticus*-infected *Rag2*^{-/-} mice. In this context, it would be interesting to determine whether changes in methylation patterns are generated in colitis induced in *Mpo*^{-/-} mice. We recently detected 5-Cl-dC in colon biopsies

from IBD patients, levels of which were in the same range of those found in *Rag2*^{-/-} mice [29], but have not yet analyzed in non-inflamed tissues from unaffected individuals for comparison.

3.1.5 Crosslinks, double strand breaks and chromosomal aberrations—Apart from small base lesions, inflammation-induced reactive chemical species can also cause more complex DNA adducts, such as crosslinks, and genomic aberrations, such as strand breaks and chromosomal rearrangements (Figure 5). Crosslinks can arise as by-products of lipid-peroxidation, such as acrolein and crotonaldehyde R, β -unsaturated aldehydes [197–199], but were also observed in an *in vitro* system using an oligonucleotide treated with NO. In the latter system, the proportion of crosslinks reached levels up to 6% of the amount of deamination products [200]. Such levels may be of pathophysiological significance, since DNA crosslinks are potentially toxic lesions; this is the mode of action of many highly toxic cancer chemotherapeutic agents. Special interstrand crosslink repair mechanisms exist that function at the interface of nucleotide excision repair and homologous recombination and are promoted by Fanconi anemia-related genes [199]. If not repaired, DNA crosslinks may interfere with the transcriptional and replication machinery as well as with chromosomal segregation, thereby potentially supporting genetic instability and tumor development. Not only DNA intra- and interstrand crosslinks may be of toxicological relevance, but also DNA-protein crosslinks are expected to have a major impact on genetic stability and gene transcription [199]. Other types of damage that affect the integrity of DNA include strand breaks potentially causing chromosomal rearrangements and aberrations. Double strand breaks (DSB) were detected upon NO delivery to cells in culture, even at sublethal doses when other types of damage products, such as deamination products, were not yet obvious, suggesting that those may be also useful markers *in vivo*. Substantial evidence has demonstrated that NO-induced DSB arise mainly through enzymatic processes during the repair of primary DNA lesions, since neither N_2O_3 nor $ONOO^-$ are able to cause DSB by direct reaction with DNA. Thus, a likely scenario includes that primary base lesions or abasic sites are repaired by BER during which single strand breaks (SSB) are introduced that may convert to DSB when occurring in close proximity to each other or when the replication fork approaches the SSB during DNA replication [136,201]. Li *et al.* reported that the formation of DSB was accompanied by increased frequencies of sister chromatid exchanges (SCE), which represent major chromosomal aberrations associated with cell transformation [136]. SCE are caused by chromosomal rearrangements occurring during homologous recombination in an attempt to repair DSB. It was found that $ONOO^-$, but not N_2O_3 , acts as a potent inducer of HR [202]. This is in line with the fact that N_2O_3 is a potent DNA deaminating agent, whereas $ONOO^-$ is a potent DNA oxidizing agent that acts mainly on DNA bases and sugar moieties. Consequently, it was shown that oxidatively-induced DNA damage exhibit a higher recombinogenic potential than nitrosative deamination damage [202]. This was interpreted to be due to the fact that DNA oxidation produces recombinogenic strand breaks by both direct attack on the sugar-phosphate backbone and indirectly during BER repair of oxidized bases, while nitrosative damage does not involve strand breaks [202]. As a consequence, such recombinogenic processes can lead to sequence rearrangements that can drive carcinogenesis during inflammation.

Support for a potential role of strand breaks during CAC *in vivo* comes from a study analyzing TNBS-induced colitis in rats [57]. Using a PCR approach, Choi *et al.* demonstrated that strand breaks were introduced in specific sequences of gene promoter regions. This was accompanied by increased phosphorylation of the histone variant H2A.X, which is considered a marker for the DSB response, in colonic smooth muscle cells [57]. These findings are consistent with gene expression studies in *H. hepaticus*-infected *Rag2*^{-/-} mice, which revealed a robust up-regulation of the DNA-damage-signaling and senescence marker *Cdkn2a* (p16) pointing to the induction of DSB in inflamed tissue of infection-induced murine colitis [27]. Usually p16 is up-regulated in premalignant lesions as a mechanism to suppress tumor development and expression is subsequently lost in invasive colon carcinomas by *Cdkn2a* hypermethylation both in spontaneous colorectal cancer and in CAC (see below) [203–206]. As observed for other damage products, DNA strand breaks and gross chromosomal aberrations were also observed in peripheral blood cells as well as in other extra-intestinal tissues, such as lymphoid organs and liver, in chemical and immune-mediated mouse models of colitis with damage levels correlating with disease severity [168,207]. This is in line with results from *H. hepaticus*-infected *Rag2*^{-/-} mice showing strong p16 induction, not only in the colon, but also in liver tissue [27]. Whether this is of any significance with regard to cancer development in tissues other than intestine needs to be evaluated, but results from *H. hepaticus*-infected *Rag2*^{-/-} mice point to a transient induction of hepatitis presumably as a consequence of colitis development.

Systemic effects of colitis on genetic instability were also observed in human patients. Significantly higher SCE levels were reported in peripheral blood lymphocytes from patients with Crohn's disease compared to control subjects, and a significant positive correlation exists between SCE levels and disease severity [208]. Human studies also revealed multiple lines of evidence for a casual relationship between genetic instability and colon cancer development. Rabinovitch *et al.* showed that chromosomal instability is present throughout the colon of patients with ulcerative colitis and high grade dysplasia or cancer [209]. More importantly, chromosomal abnormalities affected the entire colon and were even present in non-dysplastic biopsies. Analysis of chromosomal instability was sufficient to fully differentiate these ulcerative colitis biopsies from control biopsies. However, further studies are needed to assess whether these markers of genomic instability will serve as prospective indicators of cancer risk in IBD [210]. Follow-up studies revealed that chromosomal abnormalities are at least in part related to telomere shortening [210–212]. This result is consistent with the findings that oxidative stress leads to greatly accelerated telomere attrition [213]. In particular, it was shown that telomeres shorten with age almost twice as rapidly in patients with ulcerative colitis as in normal controls with extensive telomere shortening occurring within the first 8 years of disease duration [212]. In addition, epithelial cells from colon biopsies from patients with active ulcerative colitis revealed increased phosphorylation of H2A.X and higher frequencies of anaphase bridges, which are associated with non-resolved chromosomes during mitosis [210,212]. Anaphase bridges are a hallmark of telomere dysfunction and can lead to gross chromosomal rearrangements, including chromosome losses and gains. Since genetic instability precedes cancer formation, this may contribute to a mutator and genetic instability phenotype, as it is characteristic for neoplastic progression in general. The finding that genomic instability affected the entire colon of

patients with ulcerative colitis is consistent with the multifocal occurrence of tumor development under these conditions. A recent study in patients with ulcerative colitis correlated factors of inflammation, genetic instability and cellular senescence, revealing that high levels of infiltrating leukocytes were associated with increased telomere shortening, DNA damage (γ -H2A.X), and cellular senescence. This provides important evidence that senescence acts as a tumor suppressor mechanism that is abrogated during the transition from low grade to high grade dysplastic tissue [211].

Thus, these studies suggest that colitis-associated colon cancer arises in a preneoplastic environment of inflammation-driven genetic instability, apoptosis, and cellular senescence.

3.2 DNA repair and DNA damage response

To assess the functional relevance of DNA damage during colitis with respect to disease and cancer development, a useful approach is to interfere with mechanisms that repair such lesions. Several studies have addressed this issue and impressively demonstrated the functional role of multiple repair mechanisms, including mismatch repair, base excision repair, dealkylation repair, and general DNA damage response in the maintenance of colonic homeostasis and prevention of CAC.

DNA mismatch repair (MMR) is a repair mechanism active when replication errors occur, but also when damaged bases mispair with their counterparts. One of the most important proteins in MMR is MSH2, which participates in damage recognition as part of the MutS α and MutS β complex. When *Msh2*^{-/-} mice were subjected to chronic DSS-induced colitis, they developed a ~4-fold higher frequency of high-grade dysplasia and a 2-fold higher susceptibility to the development of colon tumors than controls [214]. In contrast to wild-type mice, the majority of *Msh2*^{-/-} mice developed tumors with a microsatellite instability phenotype, indicative of MMR defects. Of note, this instability appeared also in non-neoplastic tissue, presumably due to increased inflammation-induced cell proliferation [214]. Microsatellite instability was also found in patients with ulcerative colitis, in both tumor and in premalignant tissue that had no histological evidence of neoplastic change. Thus, in this setting microsatellite instability may reflect the inability of MMR to compensate for damage resulting from chronic inflammation, and may contribute to malignant cellular transformation and increased cancer risk in patients with ulcerative colitis [215]. However, as Kohonen-Corish *et al.* point out, although MMR predisposes to inflammation-induced tumor formation in mice, it is obviously not the only factor involved, since not only *Msh2*^{-/-} but also ~30% of wild-type mice developed tumors [214]. Liao *et al.* showed that in *Ogg1*^{-/-} mice, which lack the DNA glycosylase responsible for the excision of 8-oxo-dG as an initial step during BER, DSS-induced colitis leads to twice as many colorectal adenocarcinomas than observed in wild-type mice. This outcome was partially independent of potential differences in disease severity, since *Ogg1*^{-/-} and wild-type mice exhibited similar numbers of infiltrating macrophages. Importantly, quantities of 8-oxo-dG were considerably increased in inflammatory and epithelial cells in *Ogg1*^{-/-} mice [216].

Another DNA glycosylase involved in the removal of small base adducts, such as methylated bases, hypoxanthine and base ethenoadducts is alkyladenine DNA glycosylase (AAG, also known as MPG). Exposure of *Aag*^{-/-} mice to DSS-induced colitis led to a

higher accumulation of etheno adducts and oxidative base lesions in colonic DNA. This was accompanied by more pronounced colonic epithelial damage and higher cancer rates, indicating that AAG-mediated DNA repair contributes to tumor-protective mechanisms in DSS-induced colitis [175]. Impressively, when combined with genetic deletions of *Alkbh2* and *Alkbh3*, which are also involved in the repair of DNA etheno adducts, the triple knock-out mice were unable to survive a single DSS application (administered alone or in combination with AOM), indicating that AAG, ALKBH2 and ALKBH3 are crucial to maintain colon homeostasis and whole-animal survival during acute inflammatory conditions. Triple knock-out mice also exhibited decreased histopathology scores, indicating that a functional DNA damage response is important to trigger full tissue inflammation. It is important to note that DSS itself does not induced DNA damage and that the observed effects can be attributed to tissue response mechanisms. This is evidenced by the finding that when triple knockout mice were exposed to alkylating agents such as AOM and MMS alone, this did not affect their ability to survive the stimulus, highlighting the importance of inflammatory mechanisms in the observed response. The observed sensitivity of the triple knock-out mice seems to be dependent on the nature of the inflammatory trigger and the organ affected, since they were also sensitive to LPS-induced sepsis, but showed similar responses as wild-type mice to cerulein, which induces pancreatitis. This may be related to the fact that DSS colitis and LPS-induced inflammation act through TLR-signaling, while cerulein induces pancreatitis via cholecystokinin receptor signaling [217]. Changes in BER were also observed in non-cancerous tissues from patients with ulcerative colitis. Hofseth *et al* reported that levels of AAG and APE1 were elevated in inflamed tissue and that this was positively correlated with microsatellite instability indicating that proper balance of repair enzyme activities is essential to maintain genomic stability [218]. Similarly, Mutamba *et al.* showed that overexpression of AAG rendered XRCC1-deficient cells sensitive to NO-induced DNA damage [219]. ROS and RNS dependent mechanisms may also lead to inactivation of DNA repair mechanisms, since our recent study in *H. hepaticus*-infected *Rag2*^{-/-} mice revealed that inflamed colons showed globally lower expression levels of DNA repair factors [27]. Several other studies have also reported impairments in DNA repair mechanisms during inflammatory conditions [220–222]. Such changes may be caused by epigenetic silencing [221,222] or by direct inhibition via ROS and RNS-induced chemical modifications [223–227]. Furthermore, ROS and RNS-independent mechanisms may also contribute to changes in the expression levels of DNA repair factors. Kim *et al.* demonstrated that exposure of gastric cancer cell lines to co-culture with extracts of *H. pylori* led to decreased levels of MutS and MutL DNA MMR proteins in a dose-dependent manner. Reduction in MMR protein levels was caused by heat-sensitive *H. pylori* products, but the nature of these products is not known [228].

Apart from DNA repair, DNA damage signaling also contributes to the stress response during colitis. For example, *Atm*^{-/-} mice exhibit an increased sensitivity to DSS-induced colitis that was associated with elevated DNA damage levels and persistent immune activation [229]. Further, these mice displayed higher levels of tissue damage, up-regulation of pro-inflammatory cytokines, and increased levels of 8-oxo-dG, again indicating a positive feedback mechanism of DNA damage, signaling and inflammatory response [229]. Cell culture experiments demonstrated that NO activates ATM (and ATR) signaling which

triggered the activation of downstream targets such as p53. This can lead to p53 stabilization and its nuclear accumulation, where it regulates DNA repair, cell cycle progression and cell survival/death [230]. Such DNA damage response signaling was also identified in non-cancerous tissue from patients with ulcerative colitis, showing that iNOS gene expression positively correlates with p53 serine 15 phosphorylation levels and activation of p53-dependent downstream targets such as p21 [230]. Consistent with its general role as a master switch in tumor suppressive mechanisms, *p53*^{-/-} mice are prone to tumor development in a model of DSS-induced colitis [231], indicating that inactivation of p53 acts as a common mechanism of malignant transformation in CAC [232–237]. Such an inactivation of p53 and other tumor suppressors as well as activation of oncogenes can arise through DNA damage-dependent base mispairing and generation of loss-of-function as well as gain-of-function mutations leading to cellular transformation.

In the next section we will summarize initial attempts to link the formation of DNA damage products to the occurrence of genetic mutations that initiate and drive carcinogenesis.

3.3 Mutagenicity

Several studies focused on the conversion of ROS and RNS-mediated DNA damage into gene mutations thought to be a prerequisite for malignant transformation of cells [136,141,238–240]. A recent study demonstrated that when cells were exposed to NO delivered by Silastic tubing into the medium, this resulted in about 5-fold higher mutation frequencies in several cell types (TK6, AS52) at different genetic loci (*HPRT*, *TK1*) [241]. When Chinese hamster ovary (CHO) AS52 cells that carry a single genomic copy of the *E. coli* xanthine-guanine phosphoribosyltransferase (*gpt*) as a reporter gene were exposed to activated macrophages, the mutation frequency in the genetic target cell was enhanced by more than 9-fold compared to background levels. Application of the NOS inhibitor NMA and the O₂^{•-} scavenger Tiron, completely prevented this response, indicating the importance of NO[•] in the induction of these mutations. In an attempt to identify mutational fingerprints indicative of the spectrum of reactive chemical species, sequencing studies revealed that base substitutions dominate the mutation spectra, with transversion mutations (30–40%) being more prevalent than transition mutations (10–20%). Deletion mutations occurred at a lower frequency in mutants exposed to activated macrophages (20%) than in spontaneous mutants (~50%). These results suggest that small base-damage adducts, as induced by oxidation and nitrosative deamination, rather than erroneous repaired DSB cause increased mutation frequencies under these conditions. Almost all mutations occurred at guanine sites leading to G:C to T:A transversions (~30%) and G:C to A:T transitions (10–20%). Notably, this led to functional consequences as the frequency of gene inactivation doubled in cells exposed to gaseous NO[•] or to activated macrophages affecting 40% of induced mutants vs 20% of spontaneous mutants.

These results are consistent with those obtained from *in vivo* studies analyzing gastric tissue of *gpt* δ mice infected with *H. pylori* [242]. These mice harbor tandem arrays of 80 copies of the *gpt* reporter gene at a single genetic locus on chromosome 17. Mutation frequencies were significantly higher in *H. pylori*-infected female mice, which displayed a mutation spectrum consistent with the expected spectrum induced by ROS and RNS: ~4 times more

A:T to G:C transitions and ~2 times more G:C to T:A transversions than in uninfected controls. Similarly to the *in vitro* model, no increase in the frequency of deletions was observed. A notable difference, however, was the higher frequency of A:T to G:C transitions in the *H. pylori in vivo* model, which may arise from deamination of adenine, mediated by N_2O_3 leading to hypoxanthine which mispairs with cytosine; or, alternatively through ROS and RNS-induced lipid peroxidation products leading to highly mutagenic ethenoadenine [241,242]. Similar results were obtained in a colitis model using *Il-10*^{-/-} mice in combination with a transgenic *gpt* gene [243], in which the total mutation frequency in the colon of *gpt*⁺/*Il-10*^{-/-} mice was ~5-times higher than in *gpt*⁺/*Il-10*^{+/+} control mice. Similarly to the *H. pylori* model, transition and transversion mutations dominated the mutation spectrum and were around ~4-fold higher in *gpt*⁺/*Il-10*^{-/-} mice compared to controls. However, in contrast to the *H. pylori* model, the frequency of small deletions and insertions was also increased in inflamed tissue by ~10-fold, indicating organ- and disease-specific induction of mutation spectra which point to differences in the nature of reactive chemical species formed during inflammation [243]. Consistent with these results, similar mutation spectra were identified in APC and p53 tumor suppressor genes, and K-ras oncogenes dysplastic tissue of patients with ulcerative colitis [237,244,245]. These studies revealed a positive correlation between iNOS activity and increased frequencies in p53 transition mutations in colitis and colon cancer, suggesting that ROS and RNS are able to induce DNA damage products that can cause mutation in genes relevant for tumor development [237,245]. Concerning the cell-types which are at the highest risk to accumulate such mutations, computational models predict that stem cells near the base of the crypt are exposed to the highest concentrations of NO. In contrast to migrating epithelial cells, any oncogenic mutation in stem cells could accumulate over time, eventually leading to cellular transformation [72]. This is in accordance with experimental studies showing that crypt stem cells represent the cells-of-origin for colon cancer development [20].

Further studies will be required to define the exact spectrum of reactive chemical species that induce the observed mutational profiles. Mouse models with constitutive or tissue-specific deficiencies in genes that are responsible for the generation of reactive chemical species (*e.g.*, *iNos*, *Mpo*, *NADPH oxidases*, etc.) represent a possible tool to answer this question.

3.4 Microbial-induced genotoxicity

In considering causative parameters of genotoxicity, the potential contribution of the gut microbiota, which harbor up to 100 trillion bacteria, needs to be taken into account [246]. While it is beyond the scope of this review to discuss the various contributions of gut bacteria during colonic inflammation (reviewed in [247]), some studies elucidating the role of the gut microbiota on genomic stability of host cells deserve mention. Many mouse models of colitis and inflammation-induced carcinogenesis rely on the presence of an active gut microbiome, since germ-free mice do not develop colitis nor do they show significantly increased tumor frequencies in several colon carcinogenesis models [247]. Further, imbalance of the gut microbiota, termed dysbiosis, is sufficient to induce colitis in specific mouse strains, as noted for the *H. hepaticus*-infection model. To date two mechanisms have been identified regarding microbiotic factors directly contributing to genotoxicity in host

cells. Huycke *et al.* showed that the commensal microorganism, *Enterococcus faecalis*, produces substantial levels of extracellular $O_2^{\bullet-}$ and other ROS derivatives, through autooxidation of membrane-associated demethylmenaquinone and subsequent activation of host macrophages [248,249]. When mammalian cells and rats were colonized with $O_2^{\bullet-}$ -producing *E. faecalis*, significantly higher levels of DNA strand breaks were found than in control rats colonized with a mutant form, resulting in increased levels of chromosomal instability [250,251]. Some bacteria, including *Helicobacter* and pks^+ *E. coli* produce genotoxins such as cytolethal distending toxin (CDT) and colibactin, respectively. These toxins cause DNA SSB that accumulate as DSB, enhance cellular mutation frequencies and genomic instability that may promote malignant transformation of host cells [252–255]. Two recent studies suggest that colonic inflammation leads to a shift of the microbial composition towards facultative anaerobic bacteria such as *Enterobacteriaceae*, including colibactin-generating *E. coli* [246,256,257]. Specifically, Winter *et al.* showed that iNOS-derived nitrate generated during inflammation conferred a growth advantage to *E. coli* by anaerobic nitrate respiration— an effect that was not observed in *iNos*^{-/-} mice [246,257].

4 Conclusions

The studies discussed in this review demonstrate the critical role played by chemical mediators of inflammation in linking inflammation to colon cancer (Figure 2 and 6). Damage products covering the full spectrum of inflammation-related chemistries have been detected during colonic inflammation, notably in many cases at stages that clearly precede cancer formation (Figure 5). Consequently, some of these damage products may be included in a yet to be defined set of disease biomarkers to assess the individual status and cancer risk of IBD patients. Furthermore, mutational analyses and studies from DNA repair-deficient mice indicate that DNA damage products are of functional relevance in the etiology of CAC. Taken together, it is very likely that inflammation-related chemical species induce genetic and epigenetic alterations that affect molecular mechanisms involved in tumor initiation and progression during CAC (Figure 6).

Despite the large body of evidence linking colonic inflammation to DNA damage and carcinogenesis, many of the studies rely on correlative data, with few mechanistic studies that evaluate the functional relevance of inflammation-induced DNA damage. In this respect, the question of the role of DNA modifications as cytotoxic products that drive replication-dependent mutagenesis or as directly mutagenic species in their own right remains to be answered. Moreover, the dynamics of genetic and epigenetic alterations and the role of repair pathways need to be defined in greater detail. In many respects, the holes in our knowledge result from limitations of current methods to map and quantify DNA lesions. That is, methods effective on a single cell level (e.g., antibodies) often lack chemical specificity, while methods of high chemical specificity (e.g., LC-MS/MS) are currently not sensitive enough to be used in single cell analyses. This motivates the development of methods capable of mapping sites of DNA damage genome-wide, ideally in a real-time, dynamic manner. Such methods could be employed to define the whole adductome of DNA lesions in a genome-wide manner and to determine the contribution of the individual types of damage to induce gene mutations in different types and stages of IBD. A first step in this direction has been taken recently in a proof-of-principle study by employing nanopore

sequencing technology to detect guanine oxidation products in a DNA sequence context [258,259]. In addition, further progress in defining the mechanistic link between IBD and cancer will be made by large-scale sequencing analysis of genetic and epigenetic changes occurring during inflammation-induced carcinogenesis and by developing mouse models of constitutive or conditional deletions of enzymes involved in the generation of ROS and RNS, such as iNOS, NADPH oxidases, MPO, and XOR. With respect to the latter point, it is worth mentioning that, despite the finding that gene regulatory mechanisms during some inflammatory diseases show weak conservation between a specific mouse strain and humans [23], the data reviewed here demonstrate that this may not be the case for expression and activation of enzymes generating reactive chemical species, and in particular, not for the damage products that arise from these species. Indeed, many classes of macromolecular damage products arise during both murine colitis and human IBD (Table 2). Therefore, in addition to efforts to modulate immunological mechanisms to treat IBD [260], it is worth considering other factors as potential therapeutic targets, such bacterial genotoxins and enzymes producing or scavenging reactive chemical species. The fact that it usually takes decades from the onset of IBD to the first occurrence of neoplastic lesions opens a significant time window for therapeutic intervention to prevent tumor development. A detailed understanding of the chemical and biological mechanisms underlying the etiology of colitis associated carcinogenesis lays the foundation for the development of such strategies.

Acknowledgments

Declaration of interest

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ABBREVIATIONS

AOM	azoxymethane
CAC	colitis-associated carcinogenesis
GPx	glutathione peroxidases
GRx	glutaredoxins
GSR	glutathione reductase
IBD	inflammatory bowel disease
iNOS	inducible nitric oxide synthase
MPO	myeloperoxidase
NOX	NADPH oxidase
NO_x	NO oxidation products
RNS	reactive nitrogen species
ROS	reactive oxygen species

TNBS	2,4,6-trinitro benzene sulfonic acid
TxnRD	thioredoxin reductases
XOR	xanthine oxidoreductase

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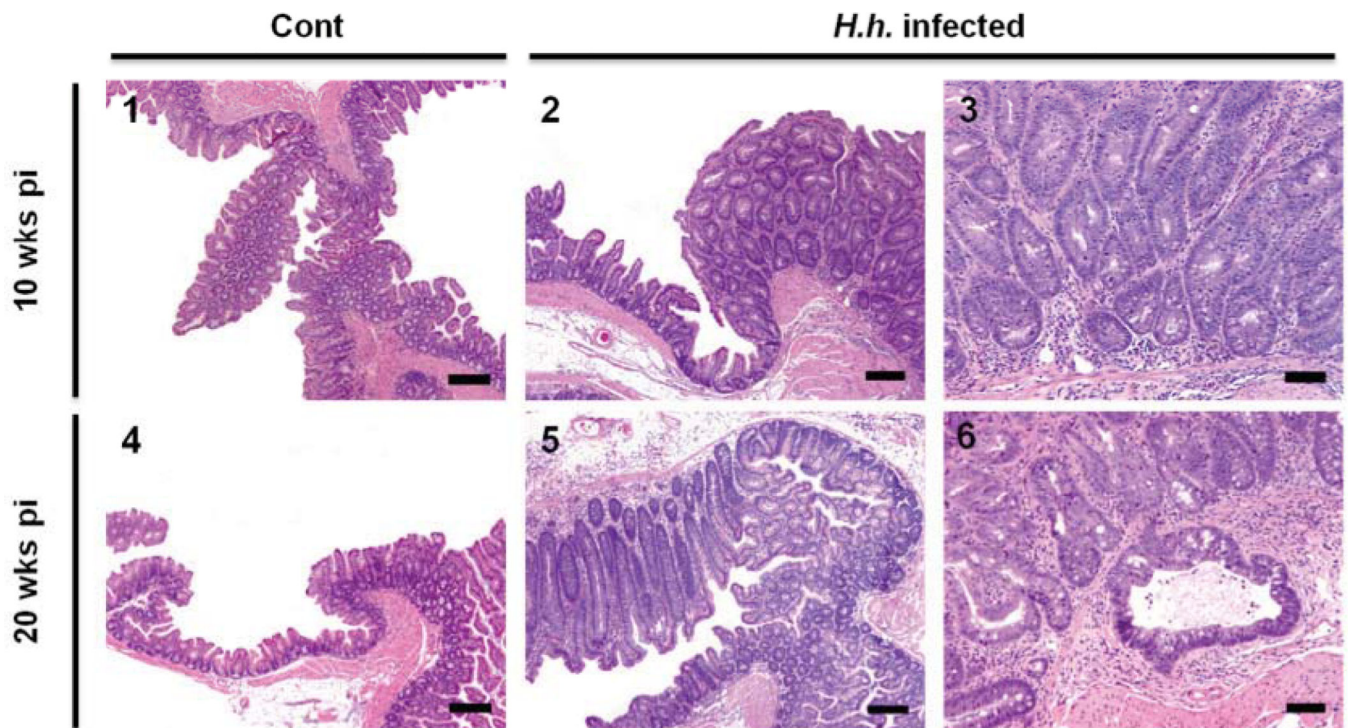


Figure 1. Colon histopathology in *H. hepaticus* infected *Rag2*^{-/-} mice

Photomicrographs of H&E-stained sections of ileocecolic junction. Representative sections from control (1 and 4) and *H. hepaticus*-infected (2–3 and 5–6) mice at 10 and 20 weeks post infection (wks pi). Images 2 and 5 show marked mucosal hyperplasia in infected mice at both time-points. Images 3 and 6 represent higher magnification photomicrographs to demonstrate the associated dysplasia and carcinoma development. Reprinted from [27]; with permission of the PNAS.

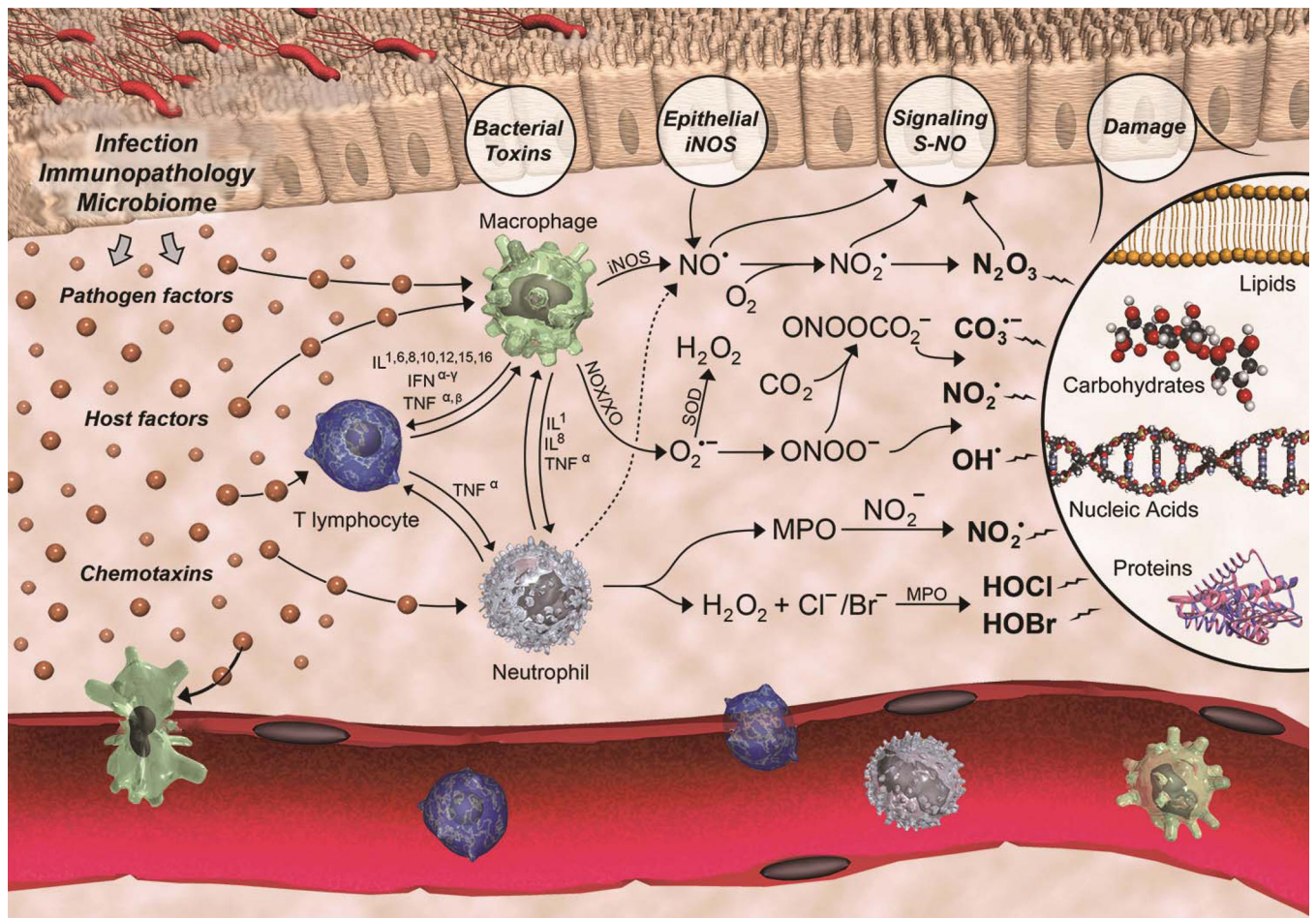


Figure 2. Chemical and biological mechanisms of inflammation [Illustration by Jeff Dixon]
 For details see text. iNOS, inducible nitric oxide synthase; MPO, myeloperoxidase; NOX, NADPH oxidase; XO, xanthine oxidase.

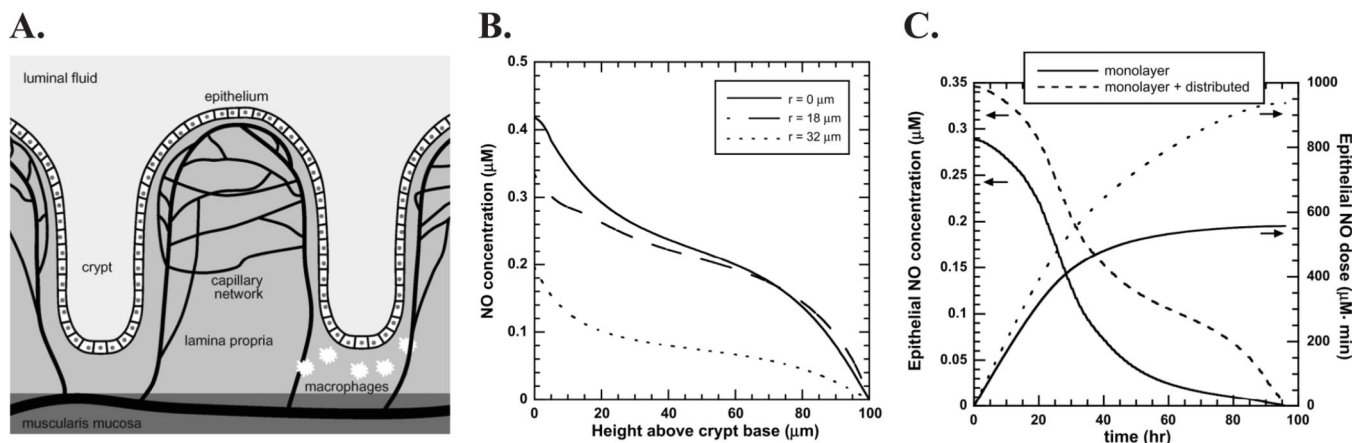


Figure 3. Computational modeling of colonic NO concentration

A. Schematic representation of colonic crypt anatomy. The inner lining of the colonic mucosa consists of three distinct layers: the *muscularis mucosa*, the *lamina propria*, and the epithelium. **B.** NO concentration as a function of height above crypt base taking into account NO synthesis by macrophages and epithelial cells. The NO synthesis rate per unit volume in epithelial cells was assumed here to be $5.6 \mu\text{M}/\text{s}$, the same as the baseline rate for macrophages. This is a conservative value; it is conceivable that rates *in vivo* are much higher. Axial concentration variations are shown for radial positions $r = 0, 18, 32 \mu\text{m}$, which corresponds to the center of the luminal fluid, middle of the epithelium, and middle of the *lamina propria* region, respectively. **C.** Time-dependent NO concentration and cumulative NO dose experienced by an epithelial cell as it migrates from the crypt base to the mucosal surface. The total time for migration was assumed to be 96 h. The ‘monolayer model’ assumes that the main source of NO is a confluent monolayer of macrophages localized in the region below the crypt base (see A.), whereas the ‘monolayer + distributed model’ assumes that macrophages are also distributed throughout the *lamina propria*. Reprinted from [72] (with permission of Elsevier).

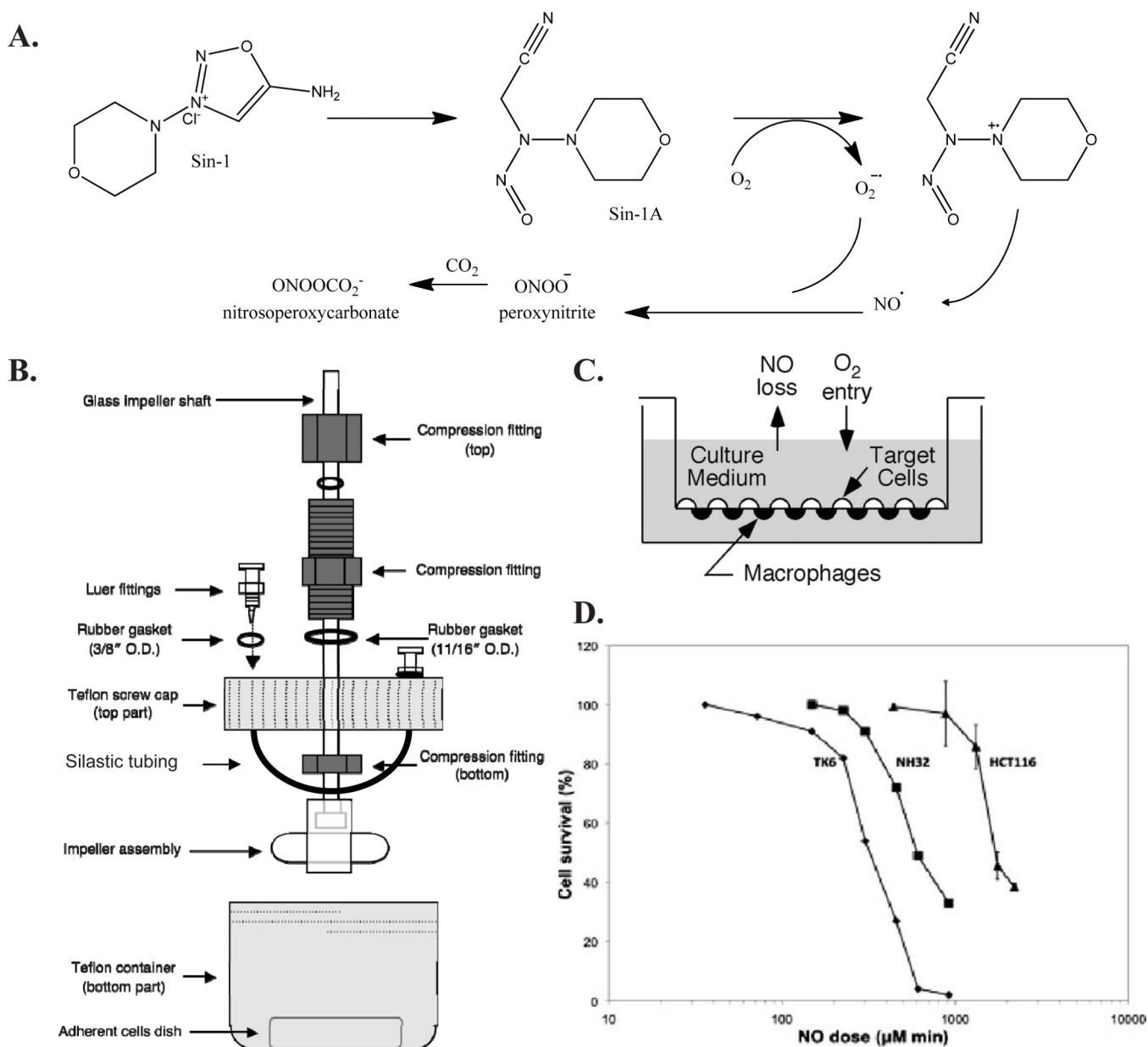


Figure 4. Examples of NO delivery systems for cell culture studies and cytotoxic profiles of different cell lines

A. Sin-1 is used as a chemical donor of NO and ONOO⁻. **B.** Delivery via NO and O₂-permeable polydimethylsiloxane (Silastic) tubing enables controlled exposure of an adherent target cell line to NO/O₂. Shown is a schematic of a typical delivery apparatus. **C.** Co-cultivation of a target cell line with activated macrophages. **D.** Cell viability in TK6 and NH32 cells at 48 h and HCT116 cells at 24 h after exposure to NO and O₂ or argon. Reprinted from [139] and [241] (with permissions of Elsevier and ACS).

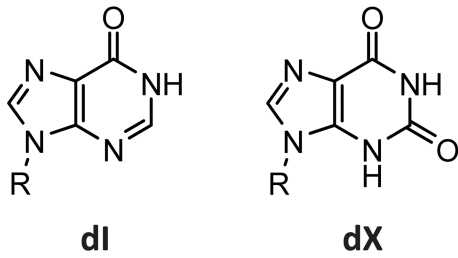
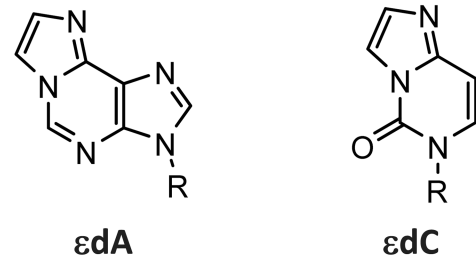
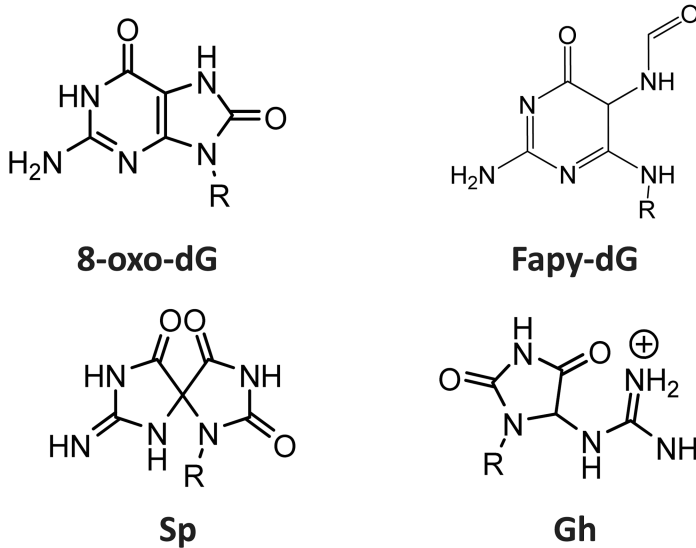
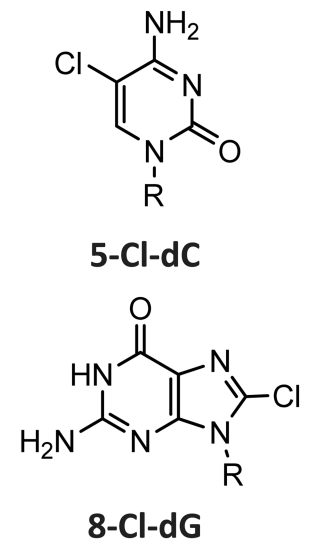
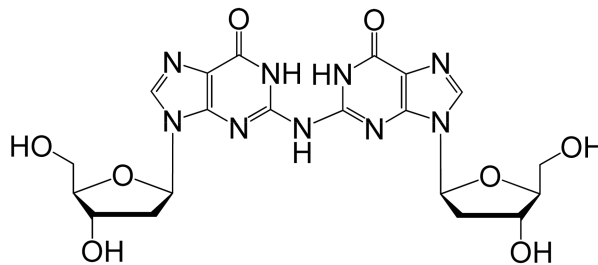
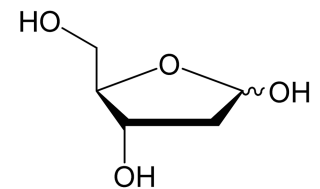
Base deamination (N_2O_3)**Lipid-peroxidation-derived products ($ONOO^-$, NO_2^+)****Base oxidation ($ONOO^-$, NO_2^+ , CO_3^+)****Base halogenation (HOCl)****DNA crosslinks****Abasic sites**

Figure 5. Overview of DNA damage products that are related to colitis-associated carcinogenesis
For details see text.

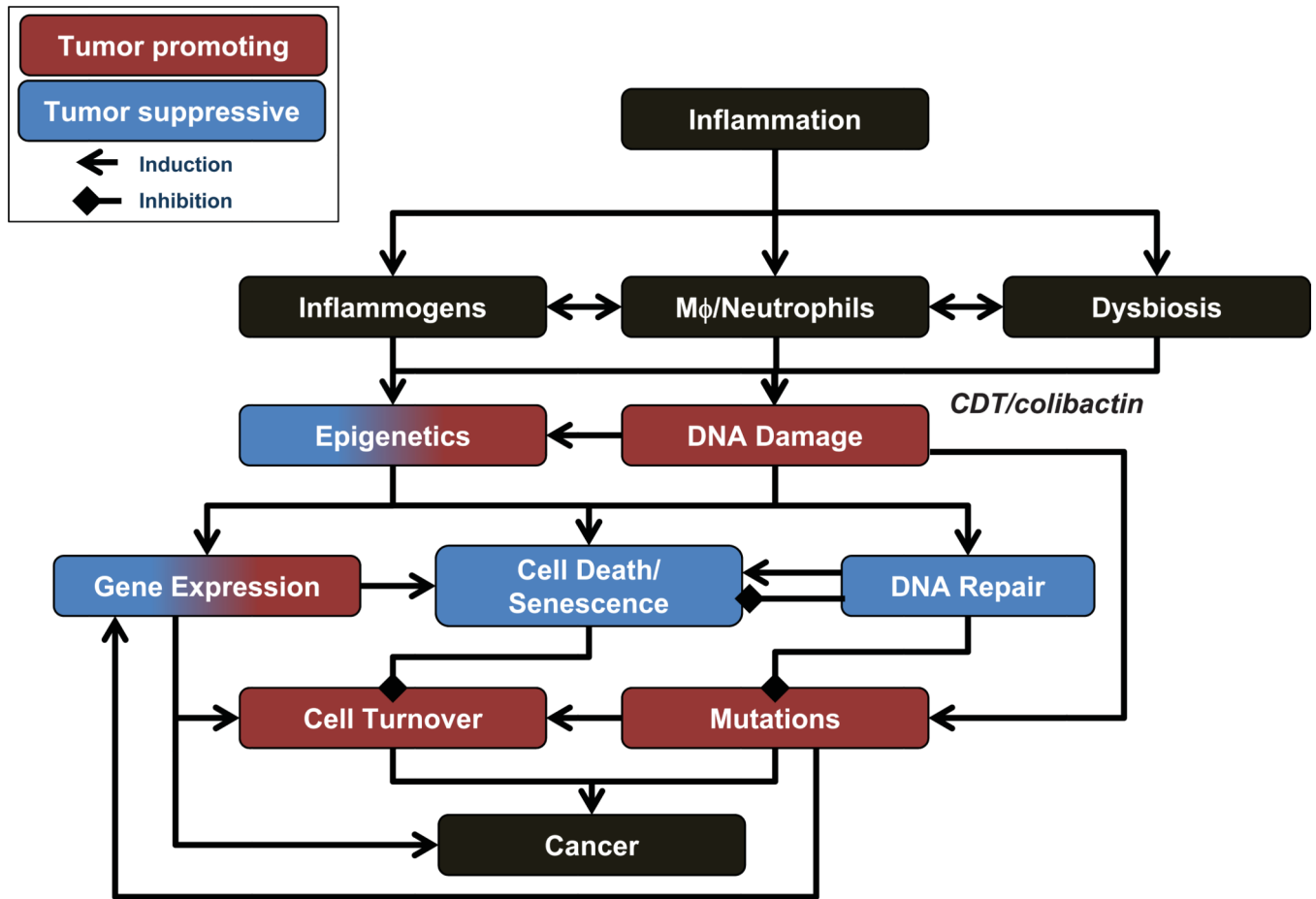


Figure 6. Overview of mechanisms that may contribute to tumor initiation and promotion during colitis-associated carcinogenesis
 Modified from [27]; with permission of PNAS.

Table 1

Common mouse models of colitis-associated carcinogenesis. For more detailed reviews on mouse models of IBD see [261–263].

Model	Specification	Mechanism	Reference
DSS	Chemical-induced (Supplementation of drinking water)	Disruption of the epithelial barrier. DSS polymers are not genotoxic, but directly toxic to colonic epithelial cells of the basal crypts. Protocols exist for acute and chronic colitis. Colitis induced within 2 (acute) to 8 (chronic) weeks. Development of dysplasia and colon cancers requires several months. In the acute phase inflammation is mainly driven by innate immunity.	[264]
DSS + AOM	Chemical-induced (Supplementation of drinking water)	Carcinogen-induced. Initial dose of AOM in combination with DSS. Bioactivation of AOM to mutagenic methyl diazonium ion. Tumor formation within 10 weeks. Useful to study the role of inflammation after carcinogen-induced tumor-initiation.	[265]
TNBS in ethanol	Chemical-induced (intrarectal administration)	Hapten-induced hypersensitivity. Activation of a T-cell-mediated response against hapten-modified autologous proteins/luminal antigens. Protocols for acute and chronic colitis. Colitis induced within 2 weeks. Inflammation dependent on innate and adaptive immunity (Th1- and Th17-type).	[264]
<i>Il-10</i> ^{-/-} mice	Genetic	Disruption of anti-inflammatory signaling. Mild disease progression. Development of a spontaneous CD4 ⁺ Th1-driven enterocolitis. Dependent on IFN- γ in the initial phase and Il12 for disease progression. Development of adenocarcinoma after ~6 months.	[266,267]
<i>Gpx1/2</i> ^{-/-} mice	Genetic	Interference with antioxidant response. GPx1/2 detoxify H ₂ O ₂ by glutathione oxidation. Development of ileocolitis between 2–7 weeks of age. Dysplasia and adenocarcinoma after ~4 months.	[120]
<i>H.h.</i> -infected <i>Rag2</i> ^{-/-} mice	Genetic + infection	Lack of adaptive immunity causes disturbed pathogen response. Induction of chronic typhlocolitis 10 weeks p.i. with first occurrence of colon carcinomas 20 weeks p.i. Since <i>Rag2</i> ^{-/-} mice lack adaptive immunity, inflammation is driven by innate immunity.	[25–28]

AOM, azoxymethane; DSS, dextran sodium sulfate; TNBS, 2,4,6-trinitro benzene sulfonic acid; GPx, glutathione peroxidase; *H.h.*, *Helicobacter hepaticus*; p.i., post infection.

Table 2

Comparison of mouse and human studies on parameters of immunological chemistry and genotoxicology.

Parameter	Mouse (model system)	Human
Infiltration of neutrophils and macrophages	↑ [37] (acetic acid), [39] (TNBS) [38] (<i>Il10^{-/-}</i>), [26,27] (<i>H.h. Rag2^{-/-}</i>), [104] (<i>C.r.</i>)	↑ [1]
Induction of NADPH ox expression	↑ [27] (<i>H.h. Rag2^{-/-}</i>)	↑ [46,47]
Induction of XOR expression	↑ [27] (<i>H.h. Rag2^{-/-}</i>)	n.c. [53]
Induction of iNOS expression	↑ [37] (acetic acid), [39,77,97] (TNBS), [78,86] (DSS), [38] (<i>Il10^{-/-}</i>), [26,27] (<i>H.h. Rag2^{-/-}</i>)	↑ [53,73,74,93,94,96,98,99,268–270]
Induction of MPO expression	↑ [79] (DSS), [39,97] (TNBS), [26,27] (<i>H.h. Rag2^{-/-}</i>), [104] (<i>C.r.</i>)	↑ [53,110]
GSH content	↓ (transient) [111] (TNBS)	↓ [113,114]
Induction of GPx2 expression	↑ [117] (DSS), [117] (TNBS), [27] (<i>H.h. Rag2^{-/-}</i>)	↑ [114,117]
Nitrotyrosine	↑ [86] (DSS), [39,97] (TNBS) [29] (<i>H.h. Rag2^{-/-}</i>), [104] (<i>C.r.</i>)	↑ [53,73,74,95,96] ↑ (in serum) [29]
Chlorotyrosine	↑ [29] (<i>H.h. Rag2^{-/-}</i>), [104] (<i>C.r.</i>)	↑ (in serum) [29]
Nitrosative DNA deamination	dI ↑ [27] (<i>H.h. Rag2^{-/-}</i>)	n.a.
Oxidative DNA damage	↑ [61,168,175] (DSS), [168] (<i>Il10^{-/-}</i>); [167] (CD45RB ^{high} CD4 ⁺ T cell → SCID transfer model), [32] (Ikkβ(EE) ^{IEC} /Apc ^{+/-} IEC) ↓ (transient) [27] (<i>H.h. Rag2^{-/-}</i>)	↑ [114,170]
Lipid-peroxidation-induced damage	↑ [175] (DSS) ↓ (transient) [27] (<i>H.h. Rag2^{-/-}</i>)	↑ [173,176–178] ↓ (in PBMC from CRC patients) [181]
Halogenation DNA damage	↑ [27] (<i>H.h. Rag2^{-/-}</i>)	Detected [29]
DSB and chromosomal rearrangements	↑ [168,207] (DSS), [168,207] (<i>Il10^{-/-}</i>), [57] (TNBS), [27] (<i>H.h. Rag2^{-/-}</i>)	↑ [208–212]

n.c., no change; n.a., data not available, CRC, colorectal cancer; *C.r.*, *Citrobacter rodentium*; *H.h.*, *Helicobacter hepaticus*