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Control and dysregulation of redox signaling in the gastrointestinal tract

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The authors declare no competing interests.

Abstract

Redox signaling in the colonic mucosa is held in an intricate balance. Potent microbicidal mechanisms can be used by infiltrating immune cells, such as neutrophils, to protect compromised mucosae from microbial infection through the generation of reactive oxygen species. Unchecked, collateral damage to the surrounding tissue from neutrophil-derived reactive oxygen species can be detrimental, and thus, maintenance and restitution of a breached intestinal mucosal barrier are paramount to host survival. Redox reactions and redox signaling have been studied for decades with a primary focus on contributions to disease processes. Within the past decade an upsurge of exciting findings have implicated sub-toxic levels of oxidative stress in processes such as, maintenance of mucosal homeostasis, the control of protective inflammation and even regulation of tissue wound healing. Resident gut microbial communities have been shown to trigger redox signaling within the mucosa, which expresses similar but distinct enzymes to phagocytes. At the fulcrum of this delicate balance is the colonic mucosal epithelium and emerging evidence suggests that precise control of redox signaling by these barrier-forming cells may dictate the outcome of an inflammatory event. This Review will address both the spectrum and intensity of redox activity pertaining to host-immune and host-microbiota crosstalk during homeostasis and disease processes in the gastrointestinal tract.

Competing interests statement

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Key points

- Immune cells, microorganisms and the epithelium all generate and respond to redox signals in the colonic mucosa during homeostasis and in disease
- Redox signals, particularly H₂O₂, are generated by the host and the gut microbiota to impede overgrowth of opportunistic pathogens; similarly, certain pathogens utilize these systems to subvert host defences
- Host responses to reactive oxygen species (ROS) produced in situ and hypoxia act in concert and opposition to regulate homeostasis in the gut.
- Host-immune and host-microbiota crosstalk can both contribute to excessive ROS production, participating in collateral damage at the tissue level.

[H1] Introduction

Mucosae are selectively permeable host surfaces, necessary for interaction with the environment and for facilitating crucial functions including gaseous exchange and nutrient absorption¹. Protecting these surfaces from both pathogenic and commensal microorganisms while maintaining immune homeostasis requires the ability to rapidly and potently induce danger signals when appropriate and to promptly neutralize these signals to limit collateral damage to the mucosa. The colonic mucosa consists of a single layer of epithelia derived from the crypt stem cell niche. As crypt stem cells proliferate, daughter cells migrate along the crypt axis, differentiating into specialized epithelia of either secretory or absorptive lineages². Absorptive enterocytes are responsible for water reabsorption, whereas secretory epithelia are tasked with mucus and antimicrobial peptide (AMP) secretion into the lumen of the gut^{2,3}. These secretions provide an essential carbon source for the microbial niche, in terms of glycosylated mucins, but they also maintain a sterile margin directly adjacent to the epithelial cells to prevent inappropriate responses to resident gut microbiota^{4,5}.

Immune and inflammatory responses within the gastrointestinal mucosae are characterized by profound shifts in tissue metabolism. These changes include the utilization of large amounts of energy and diminished availability of oxygen (hypoxia)⁶. Such shifts in tissue metabolism result, at least in part, from recruitment of inflammatory cells, particularly neutrophils (PMN) and monocytes⁷. A particularly prominent phenotype of acute inflammatory lesions within the intestine is localized accumulation of PMN, termed crypt abscesses. Given the

large amounts of reactive oxygen species (ROS) that can be generated by activated PMN, the crypt abscess represents a major signaling node for reduction-oxidation (redox) signaling⁸. Resident immune cells in the intestine, which include intra-epithelial lymphocytes and professional antigen presenting cells (dendritic cells and macrophages), are poised as sentinels to respond to host threats such as bacterial and viral infections but also contribute to homeostasis by immune surveillance and promoting a regulatory immune response⁹⁻¹¹. Most of these cell types – immune, epithelia and microorganism — are capable of eliciting and/or circumventing redox signaling with profound implications for mucosal homeostasis.

A significant result of active inflammation in the intestinal mucosae is the localized conversion of molecular O₂ to ROS and resultant hypoxia. At the tissue and cellular level, hypoxia induces an array of genes pivotal to adaptation to low O₂ states. As a global regulator of O₂ homeostasis, the $\alpha\beta$ heterodimeric transcription factor hypoxia-inducible factor (HIF) facilitates both O₂ delivery and adaptation to hypoxia^{12,13}. HIF-1 and HIF-2 (previously called EPAS) are members of the Per-ARNT-Sim (PAS) family of basic helix-loop-helix (bHLH) transcription factors. HIF activation is dependent upon stabilization of an O₂-dependent degradation (ODD) domain of the α subunit and subsequent nuclear translocation to form a functional complex with HIF-1 β and cofactors such as CBP and its ortholog p300. When O₂ supply exceeds demand, iron and O₂ dependent hydroxylation of two prolines (Pro564 and Pro 402) within the ODD of HIF-1 or HIF-2 α initiates the association with the von Hippel-Lindau tumor suppressor protein (pVHL) and degradation via ubiquitin-E3 ligase proteasomal targeting^{14,15}. A second hypoxic switch operates in the carboxy terminal transactivation domain of HIF-1 or HIF-2 α . Here, hypoxia blocks the hydroxylation of asparagine-803 so facilitating the recruitment of CBP/p300¹⁶.

A unique feature of the intestinal mucosa, particularly the colon, is the juxtaposition to large numbers of microorganisms, termed the gut microbiota. Indeed, the mammalian gastrointestinal tract is home to >10¹³ microorganisms, which approximates the number of eukaryotic cells comprising the human body¹⁷. The epithelium, a single layer of specialized absorptive and secretory cells, is all that separates this biomass from the host immune system¹⁸. A finely regulated relationship exists within the intestinal mucosa, whereby microorganisms, essential for host health, can also initiate and perpetuate mucosal disease¹⁹. Nutrient provision by microbes is one benefit to the host. In addition to aiding in digestion, microbes benefit the host through the local synthesis of short-chain fatty acids (SCFAs), including butyrate, propionate, and acetate. SCFAs can reach luminal concentrations of 130 mM in the proximal colon and function as the primary metabolic fuel for intestinal epithelial cells²⁰. Reduced production of

SCFA-producing microbial species has been associated with colonic disease, including inflammatory bowel disease (IBD)²¹⁻²³. The low-O₂ (anaerobic) conditions that enable SCFA production place unusual metabolic demands on the colonic epithelium²⁴ and are enhanced during inflammation²⁵. It is particularly notable that the microbiota is a key regulator of redox potential in the mucosa²⁶.

Aside from homeostatic and regulatory functions, ROS are well characterized to be produced and contribute to disease processes – acutely during ischemic damage, tissue injury and repair and chronically in inflammatory conditions such as ulcerative colitis, Crohn's disease and colorectal associated cancer. This Review will provide an overview of redox reactions in the gastrointestinal tract and describe how various sources of redox-sensitive pathways contribute to the function of the healthy and diseased mammalian intestine. We will also discuss exciting new findings that highlight the contributions that different intensities of redox signaling in microbial-host crosstalk have towards maintaining homeostasis or facilitating disease processes within the gastrointestinal tract.

[H1] Redox signaling in the gut

[H2] Reactive oxygen species generation

Reactive oxygen species (ROS) constitute a major group of potent antimicrobial mediators and redox signaling factors. Both the gastrointestinal mucosa and associated immune cells represent sources of free radicals, which are defined as chemical species with one or more unpaired electrons in the outermost orbital shell, making them chemically reactive²⁷. The reduction and oxidation (redox) state of the gastrointestinal tract is contingent on the balance of antioxidants (for example, heme oxygenase or glutathione, a tripeptide consisting of glutamate, cysteine and glycine) and oxidants (for example, free radicals, reactive oxygen and nitrogen species). When an imbalance in redox state occurs, owing either to increased oxidants or insufficient neutralizing antioxidants, the tissue experiences oxidative stress or nitrosative stress²⁸. In the gastrointestinal tract, a variety of reactive oxygen radicals including superoxide (O₂⁻) and hydroxyl (·OH) and non-radicals including hypochlorous acid (HOCl) and hydrogen peroxide (H₂O₂) are generated epithelia, endothelia and innate immune cells to implement mucosal defense²⁹ (Figure 1). Tissue homeostasis is influenced in a variety of ways by the redox state of the tissue, including modulation of signal-transduction pathways (e.g. HIF, NF-κB, Nrf2)³⁰ that elicit adaptive gene expression to minimize bystander tissue damage. Through reduction of disulfide bonds found in many gut peptides, redox state can also modulate the

activity of antimicrobial peptides involved in mucosal defense and cytokine secretion³¹. Of particular significance is the redox state of the ubiquitously-expressed human beta defensin-1 (hBD-1). In the oxidized state, hBD-1 exhibits a limited antimicrobial activity; however, following reduction of the disulfide bridges, hBD-1 alters conformation and displays an enhanced antimicrobial efficacy³². Indeed findings from the Wehkamp group demonstrate that the reduced form of hBD-1 is capable of forming net-like structures around bacteria to limit bacteria invasion³³.

[H2] Reactive nitrogen species

Nitric oxide (NO) is a short-lived, lipophilic and freely diffusible signaling molecule synthesized by mammalian cells with a broad spectrum of activities including regulation of blood flow, immune reactions and smooth muscle contraction³⁴. NO is generated by the nitric oxide synthase (NOS) enzymes, which convert L-arginine to L-citrulline, liberating NO as a by-product³⁵. In the gastrointestinal tract, NO functions as an inhibitory nonadrenergic noncholinergic neurotransmitter and smooth muscle cell relaxant via activation of guanylate cyclase^{36,37}. To date, three isoforms of NOS have been cloned: neuronal NOS (nNOS; NOS1), endothelial NOS (eNOS; NOS3) and inducible NOS (iNOS; NOS2)³⁸⁻⁴⁰. Interaction of O_2^- with nitric oxide (NO) leads to the formation of peroxynitrite (ONOO⁻)⁴¹. Further reactivity of peroxynitrite leads to the generation of various other NO-derived mediators termed reactive nitrogen species (RNS), including the reactive radical compounds nitrogen dioxide (NO₂) and hydroxyl radical (HO), and nonradical dinitrogen trioxide (N₂O₃)⁴¹. ONOO⁻ together with RNS are in turn responsible for nitrosylation of protein tyrosine residues, mitochondrial energy depletion, lipid peroxidation and induction of DNA strand breaks⁴². Nitrosative and oxidative stress have been implicated in a plethora of disease states, including conditions that affect the gastrointestinal tract (namely ischemia reperfusion injury and inflammatory bowel diseases)³⁰.

[H2] Sources of reactive oxygen species

Both exogenous and endogenous sources of ROS contribute to the overall redox state of the gastrointestinal tract. Endogenous sources contributing to ROS generation include the mitochondrial respiratory chain⁴³, enzymes within the mucosal epithelia and submucosal lamina propria fibroblasts and myofibroblasts such as NADPH oxidases, xanthine oxidase and immune-expressed cyclooxygenases, lipoxygenases and myeloperoxidase²⁸. Exogenous or environmental sources of ROS can also trigger oxidative stress, such as ionizing and nonionizing radiation,

chemotherapeutics, xenobiotics, heavy metals and drugs^{44,45}. Generation of ROS by cancer chemotherapeutic agents is a major contributor to the toxic side effects associated with these compounds⁴⁵. Cigarette smoke comprises >7,000 chemical compounds and oxidative agents, containing >10¹⁴ free radicals per inhalation⁴⁶. Tobacco use is known to modulate gastrointestinal diseases and active smokers display an increased risk for colorectal cancer⁴⁷ and increased severity of Crohn's disease⁴⁸. For reasons that are not completely clear, tobacco smoke appears to confer a somewhat protective influence to patients with ulcerative colitis⁴⁹.

[H1] Mitochondrial metabolism and ROS

While mitochondrial ROS (mtROS) are renowned for causing cellular damage (for example, during ischemia-reperfusion injury⁵⁰), mtROS are now thought to contribute to healthy cellular function in terms of oxygen sensing, as well as disease⁵¹. Physiological production of mtROS occurs during oxidative phosphorylation and generation of high-energy adenosine triphosphate (ATP). The tricarboxylic acid cycle is tightly regulated; however, <2% of O₂ consumption results in conversion to O₂⁻, whereby electrons leak out from the mitochondrial electron transport chain (ETC) and are aberrantly transferred to molecular oxygen⁵². Mitochondrial ETC complexes are capable of generating ROS at various sites. Complex I and II release O₂⁻ into the mitochondrial matrix⁵³, whereas manganese superoxide dismutase converts it to H₂O₂. Complex III can produce O₂⁻ within the inner membrane, but it is ejected into the intermembrane space, due to a large transmembrane electrical gradient⁵⁴. If O₂⁻ generated by the mitochondrial ETC is not efficiently converted to H₂O₂, nitric oxide radicals produce peroxynitrite (ONOO⁻), leading to subsequent irreversible nitration of proteins and enzyme inactivation⁴².

Cellular stressors such as ROS and hypoxia are hallmarks of pathogen invasion, but also reflect the local environmental fluctuations experienced by intestinal epithelial cells during active inflammation or infection⁶. There is interest in autophagy as a substantial contributor to intestinal disease mechanisms, especially IBD⁵⁵. Autophagy represents a primordial cellular degradation pathway that facilitates cell survival under conditions of metabolic stress, in which cytoplasmic targets are engulfed by a double-membrane vacuole <1 micrometer in diameter termed the autophagosome that is subsequently fused with lysosomes for hydrolase-mediated digestion⁵⁶. Considerable overlap exists between cellular stimuli for selective autophagy of damaged organelles (self) and invading microorganisms (non-self)⁵⁷. Mitophagy is a particular type of autophagy, whereby mitochondria are specifically targeted for autophagic lysosomal degradation⁵⁸. Mitophagy is a highly regulated event and some studies indicate

that the mitochondrial 18 kDa translocator protein (TSPO) is central to both regulation of mitochondrial ROS generation and the induction of mitophagy⁵⁹. Interestingly, the overexpression of TSPO in animal models of IBD have revealed that TSPO localizes with epithelial mitochondria⁶⁰. Considering the Endosymbiotic Theory, which postulates the ancient common origin between mitochondria and proteobacteria⁶¹, it is curious to speculate how a pathway such as autophagy evolved to ignore functionally competent mitochondria and their proteobacterial ancestors, but be triggered by invasive pathogenic organisms or damaged mitochondria.

[H2] H₂O₂ as a signaling molecule

Oxygen radicals have a limited range of effect due to their short-lived and highly reactive nature⁶². Specialized enzymes, such as superoxide dismutases, convert oxygen radicals to the more stable and readily diffusible H₂O₂ (Figure 1). Due to its reduced reactivity, increased half-life and ability to induce reversible protein modification, H₂O₂ can act as a signaling molecule in its own right⁶². H₂O₂ has been demonstrated to oxidize cysteinyl thiol, induce disulphide bond formation and mediate glutathionylation of cysteine or sulphoxidation of methionine residues in numerous proteins. Such modifications can alter protein activity (increased or decreased) but also represents an important antioxidant defense mechanism⁶³. In this Review we shall focus primarily on the role of H₂O₂ in mucosal-microbiota crosstalk, but it is noteworthy that other redox signaling mechanisms (e.g. nitrosylation), provide important signaling cues during host-bacterial interactions⁶⁴.

[H2] Antioxidant pathways

Regulators of the antioxidant response include enzymes that catalyze and neutralize ROS, ensuring their potent activity is short-lived to minimize collateral damage to the host tissue. Within the gastrointestinal mucosa antioxidant defense systems, superoxide dismutases and glutathione peroxidase enzymes act as detoxification pathways for ROS. Superoxide dismutases (SOD) are metal ion cofactor-requiring enzymes that catalyze the dismutation (i.e. partitioning) of superoxide anions to H₂O₂ and oxygen⁶⁵. In humans there are three SOD isoforms: mitochondrial SOD (manganese-requiring), cytosolic and extracellular SOD (both requiring copper and zinc). Mucosal injury mediated by H₂O₂ can be mitigated by SOD activity in the gastrointestinal tract⁶⁶. Indeed increased

SOD activity is associated with mucosal healing of human gastric ulcers, whereas reduced SOD correlates with increased ulcer severity ⁶⁷.

Conversion of glutathione into oxidized glutathione is performed by the glutathione peroxidase (GPX) enzyme system. In the process, H₂O₂ is enzymatically reduced to H₂O ⁶⁸. Within the human gastrointestinal tract, expression of GPX1 is ubiquitous but GPX2 is expressed specifically in epithelial cells ⁶⁹ and is postulated to protect the mucosa from transporting luminal-derived lipid hydroperoxides ⁷⁰. Deletion of either *Gpx1* or *Gpx2* in mice had no phenotypic effect, but double-knockout mice develop spontaneous colitis ⁷¹. Dismutation of H₂O₂ can also be achieved by the enzyme catalase, which converts to 2H₂O₂ to 2H₂O and O₂ ⁷². Peroxiredoxins represent another important family of thiol-specific antioxidant enzymes, designated PRDX1-6 and encoded by 6 different genes (reviewed extensively elsewhere ⁷³). It is notable that there little redundancy exists within this family of proteins, where the loss of individual peroxiredoxin lead to numerous pathologies, including haematological disorders, tumors and increased susceptibility to diseases associated with oxidative stress ⁷⁴. Somewhat surprisingly, mice deficient in PRDX2 and PRDX6 are protected from acute colitis ^{75,76}. While not completely clear, the mechanism of PRDX2-mediated protection may involve ROS-dependent stability of FoxO1 and Foxp3 regulatory T cell development.

A crucial regulator of the antioxidant response is the NF-E2 related factor 2 (Nrf2) transcription factor. Nrf2 forms heterodimers with small Maf proteins and binds to antioxidant response elements in the regulatory region of promoters of cytoprotective and antioxidant enzymes, regulating de novo transcription. Kelch-like ECH-associated protein 1 (Keap1), an adaptor subunit of Cullin 3 ubiquitin ligase, regulates the function of Nrf2 by acting as a redox sensor (reviewed ⁷⁷). Thus, antioxidant pathways provide an equally important and significant balance to redox signaling responses in the gastrointestinal tract.

[H1] Redox signaling in the immune system

Active mucosal inflammation can rapidly deplete both nutrients and oxygen in the immediate environment. For example, when activated, PMN can increase their O₂ demand by as much as 50-fold in the generation of ROS (the so called respiratory burst mediated by NADPH oxidase) necessary to kill microbes following phagocytosis ⁷⁸. By contrast, proliferating T cells only moderately increase oxygen consumption during immune responses ⁷⁹. Mucosal tissues possess both the ability to generate and attenuate redox signals; however, it is widely accepted that in the context of inflammation, the majority of radicals and reactive species are derived predominantly from the activity of

resident and infiltrating immune cells, in particular, professional phagocytes of the innate immune system, such as neutrophils, monocytes, macrophages, dendritic cells and mast cells.

[H2] NADPH oxidases and ROS

The plasma-membrane NADPH oxidase (NOX) family of enzymes are a group of paralogous enzymes, sharing common subunits. The complexes are made up of both membrane and cytosolic protein subunits that, upon activation, organize in the membrane to catalyze the conversion of molecular oxygen to superoxide anion⁸⁰. The spectrum of NOX-mediated activity ranges from potent bactericidal capacity of professional phagocytes to critical intracellular signaling in numerous cell types.

In terms of enzymatic capacity, the redox factors produced by phagocyte oxidases and peroxidases exemplify the extreme end of the redox spectrum. In addition to phagocytes expressing NOX2, fibroblasts, endothelial and epithelial cells all express enzymes that permit generation of ROS, including NOX1, NOX3, NOX4, NOX5, DUOX1 and DUOX2⁸¹ (Figure 1). While DUOX2 and NOX4 are expressed throughout the human gastrointestinal tract, NOX1 expression is highest in the distal colon where it is restricted to the cytosol, presumably to transduce intracellular signaling²⁹. By comparison, DUOX2 is expressed on the apical surface of epithelia, ostensibly enabling luminal secretion of ROS⁸². Others have examined the influence of NOX1 or DUOX2-derived ROS on *Campylobacter jejuni* infection and discovered that ROS impaired bacterial capsule formation and virulence by altering *C. jejuni* gene expression⁸³.

[H2] ROS and innate immunity

Innate immune cells, including neutrophils, macrophages and dendritic cells represent the front-line of immune surveillance and defense and generation of ROS is a crucial microbicidal mechanism used by these cells. Activation of the NADPH oxidase complex in innate immune cells elicits a rapid and potent respiratory burst⁸⁴. Defects in phagocyte NADPH oxidase function, such as in patients with chronic granulomatous disease (CGD), lead to leukocytes capable of phagocytosing but with impaired bacterial clearance⁸⁵. The hallmark of CGD is recurrent bacterial and fungal infections. Typically ~40% of patients with CGD develop IBD-like symptoms⁸⁶.

Following their recruitment to sites of inflammation, monocytes can polarize into either ‘classically activated’ (M1) or ‘alternatively activated’ (M2) macrophages, depending on the redox state and cytokine milieu of the mucosa⁸⁷ (Figure 2). Typically, TNF α and IFN γ are accepted to elicit an M1 phenotype and T helper type 2 cytokines result in M2 polarization; however, it is also apparent that macrophage phenotypes can display mixed phenotypes⁸⁸. These differentially polarized macrophages exhibit a spectrum of functionalities. The M1 phenotype is regarded as pro-inflammatory and characterized by expression of iNOS and, consequently, are an important source of RNS⁸⁹. M2 macrophages are thought to demonstrate a range of activities ranging from wound healing (release of TGF- β) to suppressing T-cell function⁴¹. Expression of the enzyme Arginase 1 by M2 macrophages depletes L-arginine, resulting in a down-regulation of the T cell receptor (TCR) ζ chain⁹⁰, impairing T lymphocyte function and resulting in immunosuppression. Aside from suppressing T cell function, ROS also contribute to regulatory T cell polarization and function^{91,92}. The exact molecular mechanisms of how ROS influence regulatory T cell function have yet to be elucidated. Taken together, the net influence of ROS in macrophage polarization might promote a state of immune tolerance as it relates to regulation of T cell function.

[H1] Host–microbial interactions and ROS

The mammalian large intestine plays host to trillions of bacteria, viruses and fungi, collectively termed the microbiota. A finely balanced mutualism exists within the intestinal mucosa, in which microorganisms, essential for host health, might also initiate and perpetuate mucosal disease⁹³. The epithelium that lies juxtaposed to the mucosal immune system serves as a selective conduit between the host and microbial world. Recognizing that both the host and the gut microbiota (both commensals and pathogens) can generate a variety of ROS, the contribution of redox signaling to such interactions has emerged as a critical interface to host–microbe interactions in the gut.

[H2] ROS and pathogen niche expansion

Similar to resident gut microorganisms, opportunistic pathogens also use redox reactions to subvert host defenses and establish a niche. One of the most studied in this regard is the invasive enteric pathogen *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*). This pathogen is associated with acute gastrointestinal inflammation and diarrhea, and elicits neutrophil chemotaxis into the mucosa⁹⁴. Invasion is achieved through two type III secretion

systems that facilitate *S. Typhimurium* to enter and persist inside intestinal epithelial cells and mucosal macrophages⁹⁴. Prior to invasion, *S. Typhimurium* must out-compete the resident gut microbiota. Some studies indicate that inflammation amplifies proliferation of luminal *S. Typhimurium*, enabling it to overgrow other microorganisms⁹⁵. In one report, the Bäumlér group demonstrated that inflammation-induced intestinal ROS reacted with luminal thiosulfate to form a new respiratory electron acceptor, tetrathionate⁹⁶. Moreover, *S. Typhimurium* express genes to enable utilization of tetrathionate as an electron acceptor that permits the pathogen to use respiration to outcompete fermenting microorganisms and establish a niche⁹⁶. The authors subsequently demonstrated that this tetrathionate-enabled respiration provided another growth advantage to *S. Typhimurium*, permitting the utilization of epithelial-derived ethanolamine under anaerobic conditions⁹⁷.

[H2] ROS and pathogen niche restriction

The role of H₂O₂ secretion into the lumen of the gut is poorly understood, but several roles have been proposed. Some studies suggest a pro-inflammatory function for DUOX-derived H₂O₂, acting as a chemotactic signal for neutrophils in a zebrafish wound-healing model⁹⁸ and a murine allergic airway model⁹⁹. Other findings suggest apical secretion of H₂O₂ into the lumen of the gut is implicated in restricting *Helicobacter felis* colonization in mice through increased bacterial oxidative stress¹⁰⁰. Another study examined the influence of NOX1 or DUOX2-derived ROS on *Campylobacter jejuni* infection and discovered that ROS impaired bacterial capsule formation and virulence by altering *C. jejuni* gene expression⁸³. During *Citrobacter rodentium* infection in wild type mice, the Knaus group discovered that NOX1 regulates DUOX2 expression in the mucosal epithelium, with a resultant decrease in both superoxide and H₂O₂ production¹⁰¹. An unexpected but intriguing finding from this study was that ablation of epithelial-derived ROS, using an epithelial-restricted Cyba-deficient mouse (absence of the obligatory NOX dimerization partner), led to protection from *C. rodentium*-induced colitis. The authors attribute their findings to an altered gut microbiota with an expansion in H₂O₂-producing lactobacilli, which exert an antimicrobial effect by release of urease, lactic acid and H₂O₂¹⁰². Pircalabioru *et al.* demonstrated through the use of catalase to degrade H₂O₂ derived from lactobacilli was responsible for attenuating *C. rodentium* virulence factors¹⁰¹. Other findings to support H₂O₂ exerting an antimicrobial function include disruption of microbial intracellular signaling, which affects antioxidant defense and polysaccharide biosynthesis¹⁰³. In the human body, L-amino acids are essential for protein synthesis; however, D-amino acids function in necessary non-ribosome-based roles¹⁰⁴. Bacteria synthesize and

secrete distinct D-amino acids into the lumen of the colon¹⁰⁵ and the Waldor group demonstrated that microbiota-derived D-amino acids upregulate expression of the host epithelial-expressed enzyme, D-amino acid oxidase (DAO), which is secreted into lumen. Oxidative deamination of D-amino acids by DAO yields H₂O₂ as a by-product and protects from *Vibrio cholerae* pathogenicity¹⁰⁶.

[H2] Resident gut microbiota and ROS

A number of studies from the Neish group have highlighted a beneficial influence of probiotic and resident microorganisms in eliciting ROS generation from epithelial sources¹⁰⁷ (Figure 1). In both *Drosophila melanogaster* and mouse models, Lactobacilli species were shown to induce epithelial-derived ROS via NOX1 activity, which stimulated epithelial proliferation¹⁰⁸. Subsequent studies by this group demonstrated dependence on the redox-sensitive transcription factor NRF2 through mechanisms that involve cytoprotection and decreased epithelial apoptosis¹⁰⁹. Further studies from this group and others have elegantly implicated a role for epithelial-expressed formyl peptide receptor (FPR), responding to microbial N-formyl-methionine-leucine-phenylalanine (fMLF), in intestinal epithelial wound healing^{110,111}. Further studies have revealed that this wound healing response occurs through oxidative inactivation of the regulatory phosphatases PTEN and PTP-PEST, with associated activation of focal adhesion kinase (FAK) and paxillin¹¹². Central to such mucosal wound healing responses appears to be the regulation of epithelial cell migration. For example, redox sensitive tyrosine phosphatases (for example, SHP2 and LMW-PTP) that are expressed at the edge of wounded epithelial monolayers are critical to the organization of focal adhesions that organize epithelial migration and wound closure¹¹³. Loss and gain of function studies have shown that both ANXA1 and NOX1-null mice show substantial deficits in mucosal wound healing responses and that ANXA1 delivery promotes wound healing¹¹². These investigators have also demonstrated that FPR/NOX2-mediated ROS generation at local intestinal tissue sites select for mucus-resident microorganisms, including *Akkermansia muciniphilia*, that accelerate epithelial wound-healing, in an intestinal epithelial NOX1-dependent fashion¹¹⁴

[H1] Consequences of redox signaling

Redox-sensitive signaling pathways are often limited by the availability of extracellular and intracellular oxygen¹¹⁵ Despite this understanding, ROS generation can occur at surprisingly low oxygen tensions. The neutrophil

NADPH oxidase, for example, is fully functional at ambient oxygen concentrations as low as 1% ⁷⁸. Such observations highlight the importance of discriminating oxygen and ROS diffusion within the tissue microenvironment, as well as the variability of oxygen availability within individual tissues. These differences often determine endpoint tissue function and define the adaptability of tissues to hypoxic stress ¹¹⁶.

[H2] Colonic tissue oxygen metabolism

The partial pressure of oxygen (pO₂) at sea level is ~145 mmHg and the alveoli of healthy lungs experience a pO₂ of ~110 mmHg ¹¹⁷. The lumen of the colon is virtually anoxic, mainly due to the microbial biomass¹¹⁸, and colonic epithelia adjacent to the lumen experience and withstand a pO₂ <10 mmHg¹¹⁹. Thus, it might be surprising to discover that epithelial stem cells at the crypt base are highly oxygenated (experiencing a partial pressure of ~100mmHg) ⁹³. Such differences are compounded by epithelial metabolism and the arrangement of the microvasculature network into counter-current blood flow dynamics within each villous structure ⁹³. Epithelia adjacent to the lumen effectively exist in a state of ‘physiological hypoxia’ ¹²⁰ and are uniquely susceptible to changes in redox state. Experimental use of oxygen-sensitive nitroimidazole compounds has enabled visualization of hypoxia in these tissues both basally and during inflammation ¹²¹. It is notable that these dyes are neither dependent on redox enzymes nor changed by the NADH and NADPH levels ¹²². This technology, coupled with immunostaining, has been used to visually reflect the oxygenation of larger solid tumors in mice ¹²³ and might even be a more reliable marker than staining for HIF-1, as it is retained in chronically hypoxic cells ¹²⁴. Such physiological hypoxia (Figure 1) is reversible by oxygenation of the colonic lumen (for example, using oxygenated perfluorodecalin) ¹²⁵.

Various factors aside from limited O₂ availability influence the activity of the PHD enzymes and HIF stabilization. Among them, H₂O₂ has been demonstrated to stabilize HIF via inhibition of the PHD2 enzyme ¹²⁶. Transcriptional activity of the HIF transcription factors regulates the genes involved in adaptive responses to hypoxia, the most widely acknowledged include angiogenesis- and glycolysis-related genes (e.g. VEGF, iNOS, GLUT1, PGK1). Less well characterized, but rapidly increasing in number, are genes with associated mucosal-protective functions that enable colonic epithelial cells to restore impaired barrier function (e.g. ITF, MUC3, CLDN1, MDR1) ¹¹⁹. Original studies using genetic loss and gain of intestinal epithelial *Hif1a* expression in mice revealed a protective role for HIF in chemically-induced colitis models that corresponded to mucosal barrier

protection¹²¹. Studies with cultured intestinal epithelial cells exposed to conditions that activate HIF have identified the regulation of a number of barrier-related protective genes¹²⁷ that have now been validated in animal models of colitis^{121,128-132} and in human-derived colonic tissue^{8,133-135}. The intestinal epithelial barrier proteins encoded by HIF target genes include those that localize to the apical surface of polarized epithelia, including mucins and mucin modifiers (for example, intestinal trefoil factor), tight junction proteins (claudin-1), antimicrobial peptides and proteins important for xenobiotic clearance¹¹⁹. Each of these components are direct transcriptional targets of HIF and contribute fundamentally to the 3D intestinal tissue architecture that forms an intact barrier during homeostasis.

[H2] Induction of epithelial HIF

Following recruitment of immune cells to the mucosa, for instance following induction of experimental colitis, hypoxia extends deeper into tissue¹²¹, a phenomenon termed inflammatory hypoxia¹³⁶ (Figure 1). One study by Campbell et al.,⁸ demonstrated that during experimental murine colitis, neutrophil influx was primarily responsible for inflammatory hypoxia. By use of a combination of neutrophil antibody depletion, hypoxia-reporter mice and NOX2 deficient (Gp91^{phox-/-}) mice, the authors demonstrated that functional NOX2 not only disseminated mucosal hypoxia but stabilized HIF within the intestinal epithelium. This HIF-signature within the epithelium resulted in an adaptive transcriptional response, that the authors coined ‘transcriptional imprinting’⁸. Biopsy samples from patients with ulcerative colitis with evidence of crypt abscess formation – a pathological hallmark with transmigrated neutrophils within the lumen of the crypt – revealed induction of HIF (monitored by increases in the HIF-target gene *GLUT1*). However, it is unclear from these studies if HIF stabilization is due to depletion of oxygen or generation of superoxide or H₂O₂, as all are capable of inhibiting the PHD enzymes^{25,137}.

Another means to stabilize HIF by inhibition of PHD enzyme function is via depletion of another crucial cofactor, iron. Some findings indicate that certain microorganisms, such as *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* can stabilize HIF in lung epithelia, via secretion of low-molecular-weight, high-affinity iron chelators, termed siderophores^{138,139}. Presumably, these factors function to chelate iron bound within the active site of the PHD enzymes, though this has not been shown conclusively. Fermenting microbiota have also been shown to stabilize HIF in the colon, via short chain fatty acid release, particularly via butyrate production¹⁴⁰. Butyrate is used as the preferred enterocyte fuel source, oxidizing butyrate to CO₂¹⁴¹. The net effect is epithelial hypoxia due to increased oxygen consumption, likely resulting in PHD enzyme inhibition to facilitate HIF

stabilization ¹⁴⁰. Indeed, a study from the Bäumlér group in 2016 demonstrated that streptomycin-treated mice exhibited a decline in butyrate-producing Clostridia, which led to increased oxygenation of the mucosal epithelium, enabling enhanced *Salmonella* expansion ¹⁴².

[H2] Inflammasome activation

The NOD-like receptor family, pyrin domain-containing protein 3 (NLRP3) inflammasome is an intracellular multiprotein complex involved in perceived cellular danger response ¹⁴³. Pathogen-associated molecular patterns (PAMPs) and host-derived danger-associated molecular patterns (DAMPs) can trigger inflammasome activation ¹⁴⁴. Stimulation of NLRP3 leads to assembly of this inflammasome complex and, ultimately, to caspase-1 activation and downstream cleavage of pro-inflammatory cytokines IL-1 β and IL-18 ¹⁴⁵. The role of IL-1 β is widely studied in autoimmune diseases; however, in gastrointestinal inflammation its involvement is not fully understood. Studies using chronic mouse models of colitis have demonstrated a role of IL-1 β in accumulation of IL-17A secreting CD4+ T helper type 17 cells ¹⁴⁶. One study in 2017 by Neudecker et al., implicated CCR2⁺ monocytes and NLRP3 activity leading to IL-1 β production in the pathogenesis of acute colitis in mice¹⁴⁷. Surprisingly, mice lacking NLRP3 are hypersensitive to experimental colitis, displaying exacerbated immune infiltration and epithelial damage, primarily due to a loss of IL-18 ¹⁴⁸. Despite the intense interest in the field, relatively little is known about how the NLRP3 inflammasome is regulated at a molecular level. Some studies suggest that autophagy can negatively regulate the NLRP3 inflammasome ¹⁴⁹. Conversely, activation of ROS, ostensibly by NADPH oxidases ¹⁵⁰ has been shown to stimulate the NLRP3 inflammasome. However, patients with CGD with NADPH-deficient macrophages display normal inflammasome activation in several studies ^{151,152}, implicating other stimuli aside from NADPH-oxidase-derived ROS. Another abundant source of cellular ROS are mitochondria, which release ROS in response to elevated metabolism, hypoxia or membrane damage ¹⁵³. The Tschopp group demonstrated that inhibition of mitophagy (encapsulation and degradation of old or damaged mitochondria via cellular autophagic machinery) results in the accumulation of damaged ROS-generating mitochondria, which leads to NLRP3 inflammasome activation ¹⁵⁴. The authors subsequently demonstrated that both ROS generation and NLRP3 activation were suppressed when mitochondrial activity was disrupted by blockade of the voltage-dependent anion channel. These findings suggest that the NLRP3 inflammasome can perceive mitochondrial dysfunction ¹⁵⁴.

[H2] Goblet cell mucus secretion

Goblet cells are specialized epithelial cells that protect the barrier from microbial invasion by secretion of a mucus hydrogel¹⁵⁵. The principal components of mucus are large mucin peptides arranged in polymeric structures. Following translation, mucins undergo extensive N-linked and O-linked glycosylation modifications and are packaged into vesicles⁴. Goblet cell-derived glycosylated mucins provide a major carbon source for the gut microbiota (reviewed elsewhere⁵). Interest has centered on understanding the regulation of mucin packaging and secretion at baseline and in response to microorganisms detection¹⁵⁶, which has led to the suggestion that goblet cells actually represent an unappreciated and distinct innate immune cell^{4,157}. Another secretory epithelial lineage, Paneth cells — which are tasked with antimicrobial peptide secretion and defense of the intestinal stem cell niche — rely on autophagy to organize secretory granules¹⁵⁸. By contrast, autophagy compromised goblet cells displayed normal mucin packaging into granules¹⁵⁹. However, a combination of autophagy and NADPH oxidase-derived ROS were found to be essential for mucin release by goblet cells (Figure 2). Patel et al. demonstrated that amphisome-like vesicles form in goblet cells following autophagosome and endosome fusion and these specialized organelles regulate mucin secretion¹⁵⁹. It was subsequently demonstrated that the NLRP6 inflammasome is crucial for promoting goblet cell mucin release in response to proximity with microorganisms¹⁶⁰. In 2016, the Hansson group proposed the existence of a ‘sentinel’ goblet cell¹⁶¹ based on proximity to the crypt entrance. This sentinel goblet cell non-specifically endocytoses and responds to TLR ligands, stimulating NOX1 or DUOX2-dependent ROS production, through downstream activation of the NLRP6 inflammasome. Moreover, via intercellular gap junctions, signals are transduced down the crypt axis to elicit mucin secretion from neighboring goblet cells¹⁶¹.

Secretion of additional mucins in response to detection of microbial proximity is obviously one approach to repel a microbial onslaught, but goblet cell hyperplasia represents an alternative mechanism. As mucus erosion and goblet cell depletion are pathological hallmarks of ulcerative colitis¹⁶², repletion of both goblet cells and their mucin granule contents is necessary for epithelial barrier restitution (Figure 3). As mentioned previously, IL-18 secretion is stimulated by ROS-mediated inflammasome activation¹⁴⁵, where some findings reveal distinct and opposing roles for IL-18 and IL-22 signaling in regulating goblet cell homeostasis. For example, Flavell and colleagues demonstrated using various intestinal epithelial-specific knockout mice to target IL-18 signaling, that excess IL-18 promotes goblet cell depletion and colitis. Moreover, IL-18 appears to suppress goblet cell differentiation markers

¹⁶³. Contrastingly, immune cell-derived IL-22 has well-recognized protective mucosal effects via promoting stem cell differentiation, mucin synthesis (mucin 2), antimicrobial peptide (RegIIIγ) and goblet cell function (Fut 2 expression) ¹⁶⁴⁻¹⁶⁶. The recently characterized type-3 innate lymphoid cells (ILC3) are the major source of IL-22 within the intestinal mucosa ¹⁶⁷. In fact, during *Toxoplasma gondii* infection, ILCs and T cells required epithelial-derived inflammasome-processed IL-18 in order to release IL-22 ¹⁶⁸. Thus, a combination of redox signaling, inflammasome activity and immune crosstalk might hold the key to homeostasis between IL-18 and IL-22 signaling and indeed mucosal-microbiota homeostasis. Moreover, IL-1β can both induce activation of ILC3 and contribute to plasticity (in concert with other factors, including retinoic acid) and reprogramming of ILC1 and ex-ILC3 to ILC3s ¹⁶⁹.

[H1] Redox signaling in intestinal disorders

[H2] Ischemia–reperfusion injury

Ischemia is defined as insufficient blood flow to tissues, resulting in disruption of cell function and ultimately necrosis. A variety of tissue insults can lead to intestinal ischemia, including trauma, stroke and atherosclerosis, and reperfusion (restitution of blood supply) following ischemia can result in aggravated tissue damage. The intestine is particularly sensitive to ischemia–reperfusion injury (I/R) ¹⁷⁰. Ischemia rapidly induces expression of cyclooxygenase (COX) and accumulation of cells expressing lipoxygenase enzymes, which are responsible for generating pro-inflammatory eicosanoids from membrane liberated arachidonic acid, such as prostaglandins and leukotrienes¹⁷¹. Constitutively expressed COX1 and the inducible isoform COX2 are responsible for catalyzing the conversion of arachidonic acid to prostaglandins ¹⁷². The primary prostaglandin studied in this context is prostaglandin E₂ (PGE₂) ¹⁷³, which elicits a bifunctional influence on the intestinal mucosa, promoting injury via vasodilatory influences on the endothelium but simultaneously conferring cytoprotection to the intestinal epithelium ¹⁷⁴. Infiltrating leukocytes expressing 5-lipoxygenase and 15-lipoxygenase leukotriene B₄ (LTB₄), produced by endothelial cells, a potent chemoattractant of neutrophils and facilitates neutrophil adhesion and degranulation ¹⁷⁵. Following reperfusion, a necessary substrate (oxygen) becomes available to enable the *de novo* synthesis of an ‘eicosanoid storm’, where in essence, bioactive lipids of the eicosanoid family become significantly amplified in their production ¹⁷⁶. This combination of lipid-mediator and neutrophil accumulation in the tissue milieu results in

another consequence upon I/RI, namely lipid peroxidation¹⁷⁷, the oxidative degradation of lipids that result in plasma membrane and organelle damage. ROS generated by reoxygenated neutrophils have long been recognized as instigators of lipid peroxidation in intestinal I/RI, resulting in barrier disruption^{50,135,170,177}. Indeed, treatment with superoxide dismutase in a murine intestinal I/RI model limits the contribution of ROS to both lipid peroxidation and mucosal permeability¹⁷⁷. Experimental strategies to circumvent the deleterious exaggerated inflammation and resultant tissue damage occurring in I/RI mostly hinge on reducing neutrophil recruitment signals and leukocyte activation¹⁷⁸. The anti-inflammatory influences of carbon monoxide, derived from endogenous heme oxygenase, might be a promising therapeutic strategy to attenuate damage from I/RI¹⁷⁹. Multiple lines of evidence have revealed that the activation of heme oxygenase effectively promotes cytoprotection and inhibits the pro-inflammatory signatures elicited from multiple cell types during intestinal I/RI¹⁷⁹. Strategies to induce heme oxygenase and carbon monoxide release are in development and include HO1 fusion proteins, small molecule heme oxygenase inducers, bilirubin, glutamine and inhaled carbon monoxide¹⁷⁹.

[H2] Ischemic preconditioning

Aside from leukocyte-derived sources of ROS, the mucosa itself can contribute significantly to redox-mediated damage during intestinal ischemia. High concentrations of ATP are released extracellularly during ischemia, which are ultimately catabolized to hypoxanthine¹⁸⁰. Concomitantly, ischemic stress results in the conversion of xanthine dehydrogenase to xanthine oxidase¹⁸¹. Following tissue reperfusion, the combination of hypoxanthine, xanthine oxidase and newly available molecular oxygen yields additional sources of tissue superoxide anion¹⁸². Although some limited therapeutic success has arisen from scavenging ROS or targeting inflammatory mediators, one of the more promising strategies to reduce I/RI is ischemic preconditioning (or hypoxic preconditioning), whereby cells or tissues are exposed to brief and intermittent periods of non-lethal ischemia. Such treatments have been shown to protect organs that experience a major ischemic event, which is best studied in the heart.¹⁸³ The mechanisms involved in ischaemic preconditioning are complex but ultimately result in reduced pro-inflammatory factors, decreased lipid peroxidation and elevated levels of natural antioxidants including glutathione, SOD and HO1¹⁸⁴. Khoury et al. identified extracellular adenosine released by hypoxic preconditioned intestinal epithelia as the major anti-inflammatory factor responsible for hypoxic preconditioning¹⁸⁵. This protective role of adenosine in ischemic preconditioning corresponded with the inhibition of NF-kappaB via deneddylation (where NEDD8 is removed from

a conjugated protein) of cullin-1 (Cul1), a component of the proteasomal degradation pathway important in the activation of NF-kappaB¹⁸⁶. It was shown that adenosine might regulate HIF through similar mechanisms; for example, a small molecule deneddylator of the cullin family proteins has become commercially available. This compound, MLN4924, is an adenosine monophosphate analog that functionally inhibits Nedd8 activating enzyme and results in the de-neddylation of Cul1 and Cul2^{187,188} and has proven to be a potent HIF stabilizer in cultured cells¹⁸⁹. In this regard, HIF might function to promote tissue ischemic preconditioning, which has been shown in some studies¹³, and small molecule stabilizers of HIF (esp. PHD inhibitors) show promise in protection from damage associated with I/RI¹⁷⁸.

Extracellular adenosine is derived from the enzymatic degradation of ATP via the action of surface apyrases (e.g. CD39) and ecto-5'-nucleotidase (CD73)¹⁹⁰. CD73 expression is increased on intestinal epithelia during hypoxia in a HIF-dependent manner, resulting in increased extracellular adenosine accumulation¹²⁷. Moreover, HIF stabilization in hypoxia was also demonstrated to decrease expression of the equilibrative nucleoside transporter (ENT)1 and ENT2, resulting in reduced uptake of extracellular adenosine providing more available for extracellular signaling¹⁹¹. Extracellular adenosine signals through activation of any of four surface G-protein coupled receptors. Activation of the A1AR or the A3AR results in decreased intracellular cAMP levels (G α i-coupled), whereas adenosine binding to the high affinity A2AAR or the low affinity A2BAR is associated with elevation of cAMP (G α s-coupled)¹⁹². The predominant receptor-mediated signaling associated with intestinal epithelial cells is A2BAR and the crystal structure of agonist and antagonist bound A2AAR has been resolved¹⁹³. The majority of evidence suggests that the induction of A2BAR by HIF translates to a strong anti-inflammatory phenotype in the intestinal mucosa, at least in part associated with barrier protection^{194,195}. These studies have shown important protective roles for A2BAR in experimental colitis¹⁹⁴ and intestinal I/RI^{196,197} that results in diminished inflammation¹⁹⁸.

A number of sources of nucleotides exist in inflamed and ischemic tissue. Many cell types actively release nucleotides, particularly in the form of ATP or ADP¹⁹². Programmed cell death (apoptosis) is associated with the generation of large amounts of ATP during ischemia and inflammation. The ATP released by apoptotic cells has been demonstrated to function as a 'find-me' signal to promote phagocytic clearance during inflammatory resolution¹⁹⁹. Other studies have shown that inflammatory cells, such as neutrophils, can release ATP in an active manner through connexin 43 hemichannels^{200,201}. Platelets release nucleotides at high concentration upon activation and are

also an important source of extracellular nucleotides during ischemia. In the intestinal mucosa, for example, platelets and neutrophils have been shown to co-migrate across the epithelium and into the lumen in the formation of crypt abscesses²⁰². As originally described by Madara et al²⁰³, this local generation of luminal nucleotides results in adenosine-mediated activation of electrogenic chloride secretion and associated water movement into the intestinal lumen. This fluid transport process provides an important flushing of the mucosal surface during ongoing inflammation.

[H2] Role of ROS in IBD

IBD includes ulcerative colitis and Crohn's disease and is characterized as a chronic inflammatory condition of the gastrointestinal mucosae in susceptible individuals⁵⁵. Ulcerative colitis and Crohn's disease exhibit distinct pathophysiology in terms of effector immune functions. Common features of IBD include abdominal pain and diarrhoea, and that susceptibility to IBD is dictated by a combination of genetic, environmental and microbial risk factors⁵⁵. The microenvironment of active IBD lesions is considered to be strongly redox active, in which ROS are considered to play an important part in both inflammatory signaling and in bystander damage to surrounding tissue³⁰.

Does excessive ROS or insufficient ROS contribute to IBD? Evidence exists to support both excessive ROS or insufficient ROS as contributing to IBD. Considering the number and functional diversity of susceptibility genes in IBD identified by genome-wide association studies, it is likely that the answer to this question depends on the individual combination of etiological factors and not merely the diagnosis of ulcerative colitis versus Crohn's disease. For instance, a study of 157 patients with CGD profiled IBD risk alleles among this cohort and concluded that defective superoxide generation in CGD is a major risk factor for IBD²⁰⁴.

As alluded to earlier, the majority of patients with CGD develop IBD-like symptoms⁸⁶. A potentially confounding issue for research in this field is the mouse models used to address the roles of phagocyte-derived ROS versus mucosal sources. Campbell et al.,⁸ using a TNBS (2,4,6-trinitrobenzenesulfonic acid) model of colitis demonstrated that *Nox2*^{-/-} mice develop substantially more severe colitis, reflected by increased weight loss, increased intestinal permeability and the failure to resolve ongoing inflammation compared to wild-type control mice. Conversely Bao et al., used the same mice in a dextran sulfate sodium (DSS) model of colitis and found no difference in weight loss or disease severity when compared to wild type controls²⁰⁵. They concluded that less tissue

damage was associated with decreased oxidative burst, though no evidence was provided for increased ROS-mediated damage. A possible explanation for the discrepancy between these studies is in the nature of the models used to ascertain the relative importance of phagocyte NOX. DSS models of colitis rely on denudation of epithelial cells, beginning with erosion of apical mucus, apoptosis of epithelia and resulting innate immune infiltrate²⁰⁶. Under such circumstances, it could be argued that phagocyte-derived ROS might not be contributing to denudation of colonic epithelia, therefore only genes or therapeutic intervention that influence epithelial viability or turnover will have an appreciable effect. By contrast, TNBS involves pre-sensitizing the host immune system to hapteneized microbial antigens, with subsequent colonic exposure to the hapteneizing agent²⁰⁶. DSS results in progressive tissue damage, extending proximally from the rectum, and incremental loss of body weight over the course of the experiment. TNBS-treated animals lose and regain weight rapidly over time and tend to exhibit skip lesions with relatively intact epithelia²⁰⁶. Moreover, immune infiltrates and inflammatory mediators differ substantially between the models²⁰⁶. As such, DSS represents a wound model, whereas TNBS represents an acute-to-chronic inflammation and resolution model. Thus, it is conceivable that Nox2^{-/-} mice do not exhibit enhanced mucosal wounding but rather, fail to resolve inflammatory insults.

Despite the dependence of IBD on genetic susceptibility and observed chronic adaptive immune responses, numerous aspects of disease progression are comparable between IBD and I/RI. For instance, proinflammatory mediators such as TNF- α and PGE₂ are implicated in both I/RI and Crohn's disease^{50,207}. Involvement of neutrophils, monocytes and leukocyte-derived ROS have been implicated in both ulcerative colitis and I/RI in the colon and intestine^{50,208}. Similarly, epithelial barrier disruption and enhanced microbial translocation are features of both IBD and I/RI^{50,209}. Studies have also suggested that shifts in the gut microbiome (dysbiosis) might contribute to both I/RI and IBD^{50,210}. One important caveat to this understanding is the observation that antibody-mediated neutrophil depletion strategies in intestinal I/RI models do not appear to influence injury endpoints²¹¹, whereas neutrophil depletion substantially enhances tissue damage in multiple colitis models²¹². Another common feature between IBD and I/RI models, is accumulation of extracellular ATP in colitis models has been demonstrated to contribute to the inflammatory process, in part via stimulation of the P2X7 receptor²¹³. Also similar to ischemic preconditioning is that extracellular adenosine appears to confer mucosal protection during colitis, principally via A2BAR signaling. Indeed, murine models of whole body and conditional epithelial deletion of A2BAR results in

more severe DSS-induced colitis associated with decreased barrier function and diminished mucosal wound healing
194,195

[H2] ROS-induced collateral tissue damage

Substantial evidence exists that collateral tissue damage, the ‘bystander effect’, might result from increased oxidative stress associated with active intestinal inflammation ²¹⁴. Implications of excessive ROS-mediated tissue damage in the gastrointestinal tract include alterations of absorptive function, barrier dysfunction and dysmotility ²¹⁵. Numerous studies have, for example, indicated malabsorption of nutrients in the intestine following I/RI and in IBD^{216,217}. Colonic epithelia, by contrast, are tasked predominantly with the reuptake of water from the faecal stream, so disruption to colonic absorption in IBD manifests as diarrhea ²¹⁸. Extensive tissue damage from excessive ROS (for example, lipid peroxidation, protein chlorination of mucosal barrier proteins) and immune mediators such as TNF- α and IFN- γ increase mucosal permeability by modulating tight junction function ²¹⁹ (Figure 3). One well-documented mechanism of barrier disruption is via induction of so-called ‘leaky’ claudin tight junction proteins, such as claudin-2 and claudin-5 ²²⁰. It is notable that increases in vascular permeability might precede increases in epithelial permeability during active mucosal inflammation (see Figure 3). Tolstanova, et al used four murine models of colitis to demonstrate that early endothelial damage resulted in perivascular edema and epithelial hypoxia, which contributed to the stabilization of HIF within the mucosa ²²¹. Evidence for early ROS-mediated endothelial damage were demonstrated at the level of electron microscopy and were consistent in genetic models of colitis, including IL-10^{-/-} and G α -i2^{-/-} ²²¹. Finally, it is likely that gut motility is affected through redox-sensitive mechanisms. For example, Brown, et al showed that enteric neuron death during active colitis was mediated by nitric oxide derived from glial cells²²² (Figure 3). Such neurotoxic activity was driven by nitric oxide influences on connexin-43 activity. Moreover, exposure of sub-mucosal smooth muscle cells to microbial lipopolysaccharide probably contributes to dysmotility through the generation of large amounts of ROS and RNS ²²³. Taken together, these studies implicate collateral tissue damage associated with oxidative stress in active intestinal inflammation.

[H1] Conclusions

In conclusion, the gastrointestinal tract represents a particularly austere environment for redox-sensitive signaling. The combination of multiple sources of reactive oxygen or nitrogen species in the setting of trillions of microorganisms requires the presence of important gatekeeper mechanisms to avoid the potential chaos that could occur during active inflammation. Just as important is the need to maintain a well poised anti-microbial environment, in large part driven by epithelial and leukocyte-derived oxygen radicals. It is notable that the profound differences in local O₂ tension within mucosal tissues and substantial increases in energy demand during inflammation provide a unique setting to understand tissue metabolism under stress. Of particular interest is the metabolic shift toward hypoxia and the associated stabilization of HIF-target pathways that associates with tissue barrier function, wound healing, autophagy and inflammation resolution. Redox signals derived from immune cells, parenchymal cells (epithelia, endothelial, fibroblasts) as well as the gut microbiota are coupled with the differing potencies, toxicities and half-lives of the redox products produced locally that require tight control for tissue homeostasis. Studies in vitro and in vivo have provided new insights toward a better understanding of productive inflammatory responses and mechanisms that promote inflammatory resolution. Also relevant is the shift in tissue redox potential that mediate collateral tissue damage and endpoint organ function. A better understanding of the basic molecular signals, transcriptional programs and the environmental clues that regulate mucosal redox state (Box 1) are likely to provide new insight toward the development of novel therapies for diseases such as IBD.

[H1] Box 1: Knowledge gaps and future research directions

- What host metabolic factors control to redox state threshold and under what conditions are they generated?
- Could microbiota-derived factors that influence redox be enriched to benefit the host in health or during disease?
- Does the low oxygen partial pressure environment of the gut provide an opportunity for drug targeting and/or drug delivery?
- How does overall tissue redox state influence acute inflammatory resolution versus progression toward chronic inflammation?
- Is innate immunity more amenable to therapeutic targeting than adaptive immunity, or vice versa?

- Is pharmacological stabilization of HIF (e.g., via PHD inhibition) a viable therapeutic option for mucosal disease?
- What pharmacological approaches best mimic ischemic preconditioning and under what circumstances might this approach benefit the host?
- For therapeutic targeting of redox pathways, how might we maximize the beneficial influence of redox signaling and minimize bystander tissue damage?

Figure 1: Host-microbial redox signaling during hypoxia.

Enzymatic utilization of molecular oxygen within the intestinal mucosa facilitates redox signaling and results in both spatial and dynamic patterns of oxygen availability. a) In the healthy intestinal mucosa, a steep oxygen gradient exists between the highly vascularized mucosa and the anoxic lumen. Thus, cells within the crypt stem cell niche normally experience higher partial pressures of oxygen (~100mm Hg) compared with the luminal-effacing epithelia (<10mm Hg) which are known to normally experience hypoxia at homeostasis. This physiological hypoxia is experienced by epithelia adjacent to the lumen and results in stabilization of the HIF transcription factor. Gut microbiota secreting short chain fatty acids (SCFA), particularly butyrate, contribute to this physiological hypoxia through increased oxidative phosphorylation. Luminal redox signaling initiated by resident microorganisms releasing D-amino acids (D-aa) stimulates the epithelium to secrete D-amino acid oxidase (DAO) into the lumen, which is subsequently yields H_2O_2 . Apical expression of epithelial DUOX2 probably results in luminal secretion of H_2O_2 , which contributes to limiting opportunistic pathogen niche expansion. Probiotic Lactobacilli up-regulate epithelial NOX1 expression, which in turn induces DUOX2. Epithelial expressed NOX1 and DUOX2, utilizing microenvironmental oxygen, generate oxygen radicals to further contribute to luminal release of H_2O_2 . b) During inflammatory hypoxia, infiltrating PMN and monocytes, expressing NOX2 generate superoxide ($O_2^{\cdot-}$), resulting in inhibition of PHD enzymes and stabilization of HIF deep into the crypt. HIF transcriptional activity induces expression of barrier protective factors – antimicrobial peptides (AMPs), Mucin 3 (Muc3) and intestinal trefoil factor (TFF3). Certain opportunistic pathogens release siderophores, sequestering iron and inhibiting PHD enzymes. Sulfur metabolism of the mucosa can be hijacked by opportunistic pathogens – H_2S is routinely detoxified to thiosulfate ($S_2O_3^{2-}$); however, high levels of reactive oxygen species within the mucosa can result in tetrathionate ($S_4O_6^{2-}$) generation, which can be utilized by *Salmonella* serotypes to provide a competitive advantage.

Figure 2: Host redox–hypoxia crosstalk in the gastrointestinal mucosa.

The two major sources of endogenous reactive oxygen species (ROS) within the intestinal epithelium originate from mitochondria and NOX1 or NOX4. a. In response to pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs), epithelia recruit and activate the Nox1/4 complex, stimulating superoxide

and hydrogen peroxide generation (sources of ROS). Both enzymatic and mitochondrial-derived ROS can trigger the activity of epithelial inflammasomes. **b.** In colonic epithelia, ROS-stimulated NLRP3 inflammasome assembly leads to IL-18 (and IL-1 β) production. Whereas excessive mature secreted IL-18 is detrimental to epithelial integrity, presence of IL-18 is necessary for IL-22 release by type 3 innate lymphoid cells (ILC3s). **c.** ILC3-derived IL-22 promotes mucosal barrier protection by inducing mucin synthesis and goblet cell function. **d.** In goblet cells, ROS triggers the NLRP6 inflammasome to elicit mucin granule secretion. **e.** Sentinel goblet cells responding to microbial triggers can signal to adjacent goblet cells to degranulate via GAP junctions. **f.** A combination of autophagic proteins, endosomes and NADPH oxidase-derived ROS are necessary for mucin granule formation in goblet cells. **g.** Both autophagy and mitophagy are induced by hypoxia. Mitophagy might decrease NLRP3 inflammasome activity, reducing processing of IL-1 β and IL-18. **h.** Inhibition of PHD enzymes by ROS or hypoxia stabilizes HIF1 α transcription factor, regulating barrier protective genes. **i.** Unimpeded or excessive ROS generation can lead to abundant maturation of IL-1 β or IL-18 or even inflammasome-mediated cell death (necroptosis/pyroptosis). **j.** Inflammasome-activation of CCR2⁺ infiltrating monocytes contribute to active IL-1 β . Mucosal IL-1 β may lead to accumulation of IL-17A secreting immune cells.

Figure 3: Reactive oxygen species collateral damage and gastrointestinal disease

During active inflammation, reactive oxygen species (ROS) and reactive nitrogen species (RNS) generated within the local microenvironment affect collateral tissues to cause damage. Activated, transmigrating neutrophils (PMN) consume large amounts of O₂ in the generation of amounts of ROS (O₂⁻, OH⁻ and H₂O₂) in the local milieu. Such O₂ consumption results in localized hypoxia and the stabilization of epithelial HIF. Epithelial HIF stabilization activates a cascade of gene transcription that promotes expression of barrier protective function genes and mucins in goblet cells. Within the lamina propria, activation of glial cell inducible nitric oxide synthase and the generation of nitric oxide (NO) results in enteric nerve cell death to result in dysmotility. Smooth muscle responses to oxidant stress include increased Ca²⁺ permeability that perpetuates intestinal dysmotility. An early event in acute mucosal inflammation within the gastrointestinal tract is increased vascular permeability and the generation of NO⁻ to promote local tissue damage.

Review criteria

PubMed was searched from 1999 to 2017 for articles using the terms: “reactive oxygen species”, “hydrogen peroxide”, “hypoxia”, “microbiota”, “mucosa” and “epithelium” alone or in combination. Articles in English were considered on the basis of their relevance to this article's topic. The reference lists of articles were crosschecked for additional references.

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Author contributions

E.L.C researched data for the article. E.L.C and S.P.C made a substantial contribution to discussion of content, wrote the article and reviewed/edited the manuscript before submission.

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