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Redox regulator network in inflammatory signaling

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Dysregulated redox signaling can exaggerate inflammation, but it is now recognized that reactive species are central mediators to both pro-inflammatory and anti-inflammatory processes. Signal regulation can occur by direct modification of proteins or lipids, or via the coordinated transfer of electrons between proteins forming a redox relay. Recently the focus shifted from reactive oxygen and nitrogen species to sulfur and carbonyl species in inflammatory signaling. Extensive interplay exists between distinct species, either within individual cells, between distinct cells, or between host cells and microbes. Reactive species act as one highly regulated network regulating inflammatory decision making of the host. Selected examples, mainly in innate immune cells, will be presented, emphasizing the interconnected nature of reactive species during inflammatory signaling.

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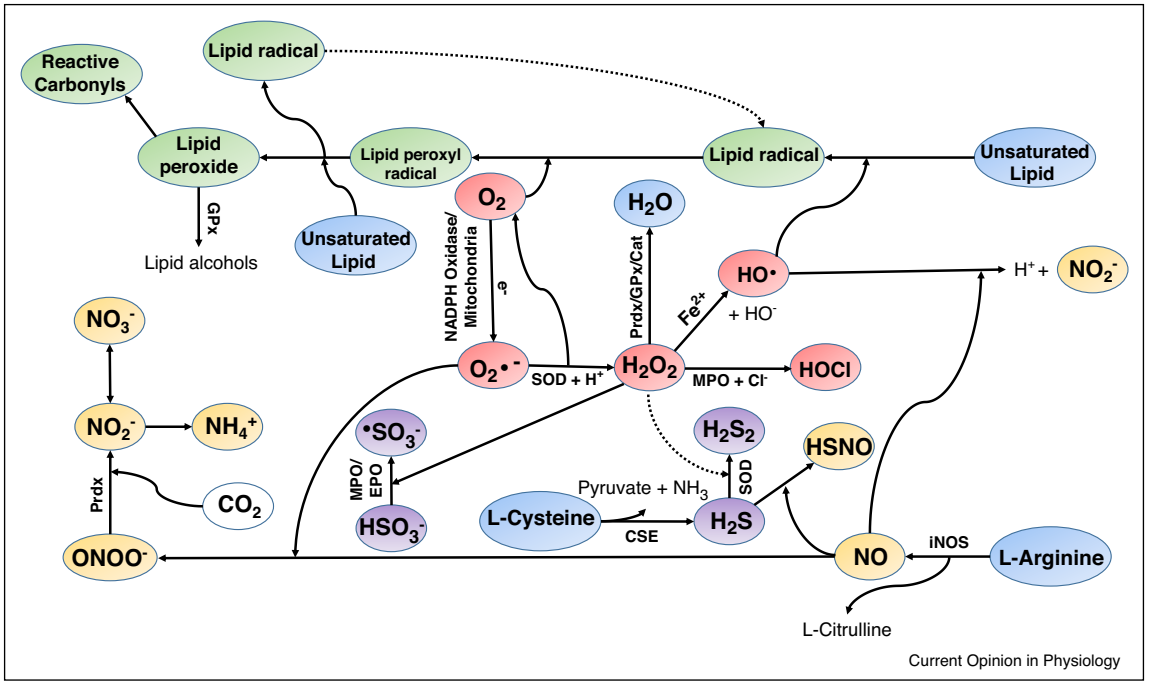
Interactive networks in redox signaling

Biological systems utilize oxidation–reduction (redox) reactions to modulate their responses to environmental cues. When considering redox processes in cell signaling, emphasis is often placed on Reactive Oxygen Species (ROS) or Reactive Nitrogen Species (RNS). These species are vital; however, they rarely act alone. Signaling via ROS, RNS, Reactive Sulfur Species (RSS) and Reactive Carbonyl Species (RCS) should not be considered in isolation, but rather as one all-inclusive reactive system governing cellular responses. This is not only important in intracellular or cell-to-cell signaling, but also in host–microbe interactions involving the innate immune system or at mucosal barriers, where reactive species are generated, converted and metabolized by both mammalian cells and bacteria.

Superoxide ($O_2^{\bullet -}$) and superoxide derivatives are central to redox signaling. NADPH oxidases and mitochondrial complexes I and III are notable $O_2^{\bullet -}$ sources. As a next step, superoxide dismutases (SOD1-3) accelerate $O_2^{\bullet -}$ dismutation to hydrogen peroxide (H_2O_2), which can be converted into hydroxyl radicals ($HO^{\bullet}$) and hydroxyl ions (HO^-) via the Fenton reaction. Neutrophil myeloperoxidase (MPO) uses phagosomal H_2O_2 to generate hypochlorous acid (HOCl). $O_2^{\bullet -}$-mediated oxidation of NO produces peroxynitrite ($ONOO^-$), the archetypic RNS. H_2O_2-mediated, SOD-catalyzed oxidation of H_2S yields persulfides, while H_2O_2-mediated, MPO/EPO-catalyzed oxidation converts (bi)sulfite (HSO_3^-) into the sulfur trioxide anion radical ($^{\bullet}SO3^-$) and other sulfur centered radicals, a second class of RSS [1–3]. ROS and RNS are both involved in lipid peroxidation. Lipids are targeted at polyunsaturated fatty acid residues by radicals initiating the lipid peroxidation chain, resulting in lipid peroxide and lipid radical production. RCS are highly reactive carbonyl derivatives of lipid peroxides (Figure 1). Although ROS are central to the production of certain RNS, RSS, and RCS, ROS conversion can itself be regulated by NO and H_2S. For example, NO is a competitive inhibitor of catalase, preventing catalase-mediated degradation of H_2O_2 to H_2O [4]. An indirect antioxidant function of H_2S has been reported. Matsui *et al.* observed the heme-degrading function of heme-oxygenase (HO) being maintained under hypoxic conditions in the presence of H_2S [5•]. Sulfur donation from H_2S partially replaced the requirement for oxygen transfer, resulting in the production of sulfur-containing bilirubin (SBR) in the place of native bilirubin. The authors proposed that the antioxidant properties of bilirubin could be retained by the production of SBR in hypoxia. As the H_2S generator cystathionine beta-synthase requires heme as a co-factor, maintenance of HO-1 function maybe a mechanism of H_2S self-regulation when oxygen supply is decreased.

Many immune signaling pathways are regulated through redox reactions. For example, circulating HMGB1, an endogenous ligand for Toll-like receptor 4 (TLR4), requires thiol group oxidation and disulfide bond formation before recognition by TLR4 [6]. Signal regulation, either inhibitory or stimulating, also occurs through the oxidation of enzymes. The phosphatase PTEN undergoes reversible inactivation upon H_2O_2-mediated oxidation [7]. Furthermore, cysteine oxidation has been proposed to promote the unfolding and subsequent activation of the non-receptor tyrosine kinase Src [8]. An indirect mechanism of H_2O_2-mediated signaling has also been suggested. Sobotta *et al.* reported a redox relay

Figure 1



Reactive species form an interactive network.
Selected oxidation, conversion and degradation pathways of reactive oxygen, nitrogen, sulfur and carbonyl species are shown. For example, superoxide ($O_2^{\bullet -}$) oxidizes nitric oxide (NO), while hydrogen peroxide (H_2O_2) oxidizes hydrogen sulfide (H_2S) and bisulfite (HSO_3^-). The reaction between H_2S and NO produces thionitrous acid (HSNO). Hydroxyl radicals ($HO^{\bullet}$) react with both unsaturated lipids and NO. Antioxidant and oxidant converting enzymes are promiscuous in their specificity: glutathione peroxidase (Gpx) can convert hydrogen peroxide (H_2O_2) to water (H_2O) and can also convert lipid peroxides to innocuous lipid alcohols; superoxide dismutase (SOD) accelerates the dismutation of H_2O_2 and can catalyze the H_2O_2-mediated oxidation of H_2S; myeloperoxidase (MPO) can convert H_2O_2 into hypochlorous acid (HOCl) and catalyze the H_2O_2-mediated oxidation of HSO_3^-. Additional abbreviations used: Cl^-, chloride anion; CSE, cystathionine gamma-lyase; EPO, eosinophil peroxidase; H^+, proton; HO^-, hydroxyl ion; H_2S_2, persulfide; iNOS, inducible nitric oxide synthase (NOS2); NADPH, nicotinamide adenine dinucleotide phosphate; NH_4^+, ammonium ion; NO_2^-, nitrite; NO_3^-, nitrate; $ONOO^-$, peroxynitrite; O_2, molecular oxygen; Prdx, peroxiredoxin; SOD, superoxide dismutase.

where concomitant oxidation events were shuttled from protein to protein [9]. H_2O_2 oxidized cysteine to sulfenic acid in peroxiredoxin 2, which promoted the formation of a disulfide-bonded peroxiredoxin 2-STAT3 intermediate and subsequent STAT3 oligomerization, leading to attenuated transcriptional activity. Further modification of sulfenic acid residues can occur in the presence of glutathione, leading to *S*-glutathionylation of proteins, which may alter protein–protein interactions and intracellular localization. Actin, mitogen-activated protein kinase (MAPK) phosphatase 1 (MKP-1), 14-3-3ζ are examples of *S*-glutathionylated proteins identified in metabolically stressed monocytes [10••]. In addition, oxidative modification of tyrosine residues and *S*-nitrosylation are known redox-mediated mechanisms modulating signal output.

Reactive species released by host cells modify mucosal environments, and interact with NO, H_2S and H_2O_2 generated by resident intestinal microorganisms. Specialized bacteria including *Desulfovibrio* and *Desulfobulbus* produce H_2S via the reduction of sulfates, while other bacteria such as *Escherichia coli*, *Salmonella enterica*, *Clostridia*, and *Enterobacter aerogenes* contain desulfhydrases and can generate H_2S by desulfhydrating cysteine and methionine [11,12]. Lactobacilli and some other bacteria generate H_2O_2, which modifies epithelial cell signaling and is required for their protective role in intestinal infection and inflammation [13,14], while *E. coli* and *Lactobacillus plantarum* utilize nitrate and produce NO in conditions similar to those found in the intestine [15]. There is significant redox interplay between the host and microbiota in the gastrointestinal tract. Bacteria can stimulate H_2O_2 release by the NADPH oxidases NOX1 and DUOX2 [16,17], which will modulate host responses and bacterial community structure. In the inflamed gut host-generated $ONOO^-$ is converted into NO_3^- upon reaction with CO_2, and an abundance of NO_3^- shapes the composition of the microbiota, which in turn alters host responses [18,19]. NO in the intestinal lumen can interact with H_2S and other RSS [20], altering the chemical environment in the gut. Furthermore, the virulence of pathogens can be downregulated by H_2O_2 produced by

intestinal epithelial cells through intrabacterial oxidation reactions targeting phosphotyrosine signaling [21••].

ROS, RNS, RSS, and RCS are generated, converted and degraded on an ongoing basis. Their respective roles are intertwined, affecting cellular and microbial responses through interactive pathways. The following chapters will focus on selected examples that emphasize the interconnected nature of reactive species during inflammatory signaling.

Redox signals contributing to macrophage differentiation and polarization

The classification of macrophages (MΦs) into microbicidal, pro-inflammatory M1 and tissue remodeling, resolving M2 phenotypes is mainly based on the group of stimuli promoting a defined phenotype *in vitro*. In pathological conditions the situation is more fluid, with mixed phenotypes coexisting in the complex progression from monocyte maturation to interaction with immune cells and pathogens up to the final steps of initiation and resolution of inflammation [22]. Macrophage polarization, heterogeneity and plasticity, and their ability to re-polarize in response to changes in the microenvironment are highly dependent on redox processes involving NO and mitochondrial and NOX-derived ROS.

One of the characteristics of various macrophage subsets is the differential use of L-arginine, which is metabolized by iNOS/NOS2 for NO generation in M1 MΦs and tumor-associated MΦs (TAMs), and by arginase-1 to produce polyamines and L-proline in classical M2 MΦs. NO and its derivatives (nitrosothiols, peroxynitrite) constrict mitochondrial respiration by inactivating various respiratory complexes [23], thereby preventing repolarization of M1 MΦs to M2 MΦs. Inhibiting NO production with the iNOS inhibitor 1400 W improved mitochondrial respiration and metabolic reprogramming toward M2 MΦs [24••]. Since M1 MΦs rely on glycolysis for ATP synthesis, the mitochondrial ETC is repurposed to produce elevated ROS by succinate dehydrogenase (SDH/CII), further driving the M1 phenotype [25]. Inhibiting SDH by dimethyl malonate reduced ROS production and promoted the anti-inflammatory phenotype. Increased mitochondrial ROS in intestinal epithelial cells and subsequent activation of the NF-kB pathway indirectly induced preconditioning in favor of an anti-inflammatory macrophage phenotype with increased recruitment of M2 MΦs (F4/80+ and CD206+ cells) [26]. Mitochondrial ETC architecture and SDH activity can also be altered by redox signal(s) originating from phagosomes. ETC adaptation was dependent on engagement of bacteria with TLR4 and was mediated by the NADPH oxidase NOX2 and the tyrosine kinase Fgr [27•]. Monocyte to macrophage differentiation and M2 MΦ polarization also require NADPH oxidases. Intracellular H_2O_2 generation by M-CSF-stimulated bone marrow-derived monocytes was dependent on two Nox isoforms [28]. Combined deletion of Nox1 and Nox2 in mice impaired MAPK activation, delayed wound healing and restricted tumor formation, while the inflammasome response and IL-1β secretion were not affected.

Phagosomal H_2O_2 production leads to the activation of the membrane bound transient receptor potential melastatin 2 (TRPM2) channel, which promoted cation efflux and together with H^+ influx decreased the phagosomal pH, thereby augmenting bacterial killing [29]. TRPM2 activation at the plasma membrane also improved bacterial clearance by activating cytokine production and inducing phagosome–lysosome fusion [30,31]. In contrast, Beceiro *et al.* found that ablation of TRPM2 in mice promoted M1 MΦs polarization and *Helicobacter pylori* clearance, while increasing inflammation [32]. These findings suggest that TRPM2 may be involved in different phases of infection that may depend on the pathogen encountered. NOX2-dependent $O_2^{\bullet-}$ production in MΦs was also required for maturation and proteolytic activity of efferosomes, as delayed proteolysis and enhanced cross-presentation of apoptotic cell-derived antigens was observed in CGD macrophages [33•].

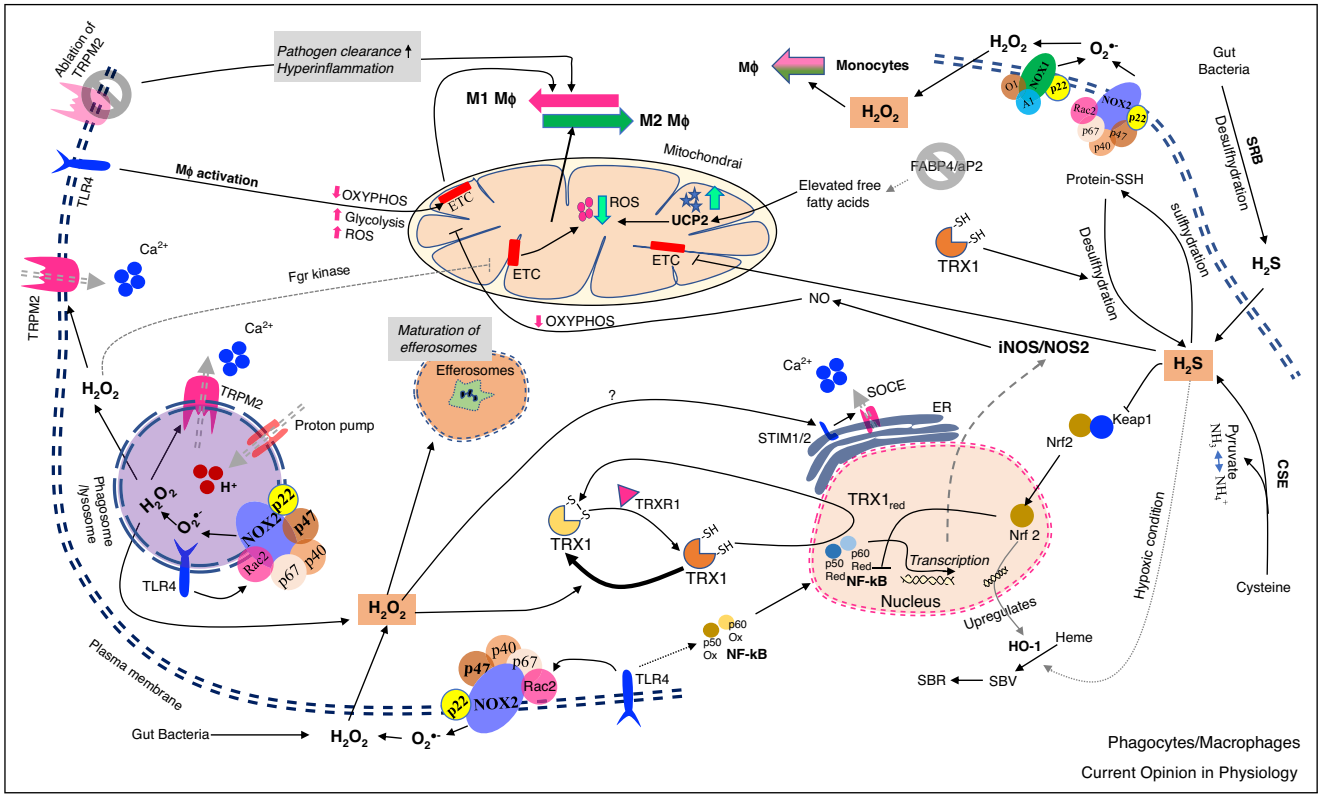
In obesity and related metabolic disorders, inflammatory macrophage accumulation and ER stress-mediated ROS generation in visceral adipose tissue drive inflammation by increasing pro-inflammatory cytokine and chemokine secretion [34]. Recent reports linked the fatty acid binding protein FABP4/aP2 to pro-inflammatory redox processes. Loss of FABP4/aP2 led to increased uncoupling protein 2 (UCP2) and antioxidant protein expression, and attenuated NF-kB signaling. These changes improved mitochondrial function, diminished H_2O_2 production and induced a phenotypic switch toward the anti-inflammatory state [35–38]. The FABP4/aP2 knockout mouse model has been extensively used to understand the molecular mechanisms linking metabolic diseases and inflammation. A schematic representation of redox signaling networks operating in macrophages and extracellular redox signals targeting macrophages, based on selected studies, are shown in Figure 2.

Input from several reactive species shapes mitochondrial metabolism and the macrophage phenotype, thus contributing to macrophage effector functions. An imbalance in M1 MΦs versus M2 MΦs can be observed in pathological conditions including inflammatory bowel disease (IBD), where M1 MΦs accumulate in the *lamina propria*, contributing to chronic inflammation and barrier defects [39]. Modifying MΦ maturation and polarization with immunotherapy as potential therapeutic option has been recently reviewed by Funes *et al.* [40].

Neutrophil redox signaling and inflammation in neutrophils

In the past neutrophils were considered to be short-lived cells, mainly involved in phagocytosis, microbial killing,

Figure 2



A schematic representation of redox signaling networks modulating macrophage function during inflammation.
The spatiotemporal generation, interaction and conversion of various reactive species regulates mitochondrial metabolism and gene expression during inflammation to carry out macrophage effector functions. Abbreviation are: CSE, cystathionine gamma-lyase; ETC, electron transport chain; FABP4/aP2, fatty acid binding protein 4/adipocyte protein 2; HO-1, heme oxygenase-1; Keap1, Kelch like ECH associated protein 1; MΦ, macrophage; Nrf2, nuclear factor (erythroid-derived 2)-like-2 factor; SBR, sulfur-containing bilirubin; SBV, sulfur-containing biliverdin; SOCE, store-operated calcium entry; SRB, sulfate-reducing bacteria; STIM, stromal interaction molecule; TRX1, thioredoxin 1; TRXR1, thioredoxin reductase 1; TRPM2, transient receptor potential melastatin 2; UCP2, uncoupling protein 2.

and pro-inflammatory activities. However, it is now evident that these cells live longer, play a vital role in inflammation by releasing pro-inflammatory as well as resolving mediators, and interact with various other immune cells. NOX2 and MPO are pivotal and central to disparate functions (pro-inflammatory and anti-inflammatory) attributed to neutrophils.

ROS generation has been associated with inflammation, oxidative damage and tissue injury, but in CGD, an inherited immunodeficiency due to inactivating NOX2 complex mutations, hyperinflammation occurs. ROS-mediated negative regulation of cytokine production has been linked to reduction–oxidation regulation of the transcription factor NF-kB. Decreased ROS generation as well as enhanced NF-kB activity and inflammation were observed in LPS-challenged mice deficient in the Nox2 complex component $p47^{phox}$ [41]. A recent study reported a new mechanism triggered by NOX2 in neutrophils and monocytes that may explain how ROS generation dampens pro-inflammatory signaling. Endotoxin-induced redox modification of a thioredoxin 1 (TRX1)/$p40^{phox}$ complex excluded TRX1 from the nucleus. Reduction by thioredoxin reductase 1 permitted nuclear translocation of TRX1, where it facilitated NF-kB transcriptional activity leading to inflammatory gene upregulation. In absence of NOX2-generated superoxide ROS, reduced TRX1 accumulated in the nucleus potentiating NF-kB binding [42••]. Further, a role for the calcium sensors STIM1 and STIM2 in distinct but cooperative regulation of neutrophil pro-inflammatory functions was reported [43]. Redox processes, in particular *S*-glutathionylation, modulate structural changes and interactions in STIM and ORAI proteins [44], a machinery regulating the store-operated calcium entry pathway. STIM1 was required for phagocytosis and neutrophil ROS generation by calcium-dependent agonists without directly affecting phorbol ester-induced Nox2 complex assembly, while STIM2 mediated cytokine production via calcium-dependent regulation of NF-kB [43].

Nox2-derived H_2O_2 was also essential for actin polymerization dynamics through redox modification of cysteine residues in actin monomers [45], for neutrophil degranulation and for NET formation [46]. H_2O_2 is converted into HOCl by MPO in the presence of chloride ions, and both ROS have been implicated in NET formation depending on the type of stimulus [46,47]. Phorbol ester requires both, the oxidative burst ($O_2^{\bullet-}/H_2O_2$) and HOCl for NET formation, while bacteria-induced NETs formation is dependent on NOX2, but independent of MPO activity. The detailed mechanism of how these different oxidants activate diverse signaling pathways to achieve a common result (NET formation) is not fully understood. Formation of secondary ROS (e.g. HOCl) in the phagosome promotes lipid peroxidation and generates RCS (e.g. HNE, ONE, MDA), which results in carbonylation of phagosomal and cytosolic proteins, namely GSH and calprotectin [48]. Calprotectin levels are increased in many inflammatory conditions and are used as marker for neutrophil presence in the active phase of IBD [49,50]. Further characterization of modified products released by neutrophils in pathological conditions will contribute to our understanding of neutrophil activation/deactivation cycles.

Several connections between RSS and neutrophil ROS exist. H_2S acted as anti-inflammatory mediator in lung injury, where it limited LPS-induced neutrophil transmigration by downregulating the chemotactic mediator MIP-2 and its receptor CXCR2, and suppressed ROS production, possibly by inhibition of MAPK pathways and calcium channels and by upregulation of catalase [51]. In contrast, other studies have shown enhanced neutrophil migration due to the opening of ATP-sensitive potassium channels by H_2S [52,53]. During the acute phase of inflammatory bowel disease neutrophil recruitment and luminal $O_2^{\bullet-}/H_2O_2$ and $ONOO^-$ release can alter bacterial community structure via H_2O_2 signaling [21••], nitrate respiration [19], and by oxidation of bacteria-derived H_2S to sulfite [54]. Sulfite concentrations in a food-safe range exhibited bactericidal effects on several *Lactobacillus* species *in vitro* [55].

Re-emergence of reactive sulfur species: role in inflammatory signaling

Cystathionine gamma-lyase (CSE) and cystathionine beta-synthase (CBS) metabolize cysteine, providing an endogenous H_2S source in cells. In addition, TRX1 was capable of replenishing the endogenous H_2S pool by facilitating protein *S*-desulfhydration [56••]. Certain gut bacteria can also produce H_2S either by sulfate reduction or by desulfhydration of sulfur-containing amino acids.

H_2S is predominantly considered anti-inflammatory. When supplied with exogenous H_2S, macrophages showed decreased ox-LDL-induced and LPS-induced NF-kB transcriptional activity and decreased TNFα secretion, while CSE knock-down increased the pro-inflammatory response [57,58•]. In addition, monocytes exposed to H_2S during mycoplasma infection showed reduced translocation of the NF-kB p65 subunit and a reduction in MCP-1 release [59]. As NF-kB was reported to regulate the transcription of NADPH oxidases under certain conditions [60,61], it seems plausible that elevated H_2S levels may reduce expression of a particular NADPH oxidase indirectly via NF-kB, resulting in decreased ROS generation. H_2S was reported to suppress glucose-stimulated Nox4 expression in renal tubular epithelial cells [62] and angiotensin II-induced Nox4 expression in cardiac fibroblasts [63].

ROS sources in addition to NOX can be regulated by H_2S. Castellano *et al.* reported H_2S-mediated inhibition of xanthine oxidase (XO) activity and decreased mitochondrial ROS production in macrophages [64]. Others also described downregulated mitochondrial activity, in particular inhibition of mitochondrial complex IV, upon exogenous H_2S treatment, but observed increased XO activity in cardiomyocytes [65]. While there may be cell type-specific effects, *S*-sulfhydration of cysteine residues in XO was required for its enzymatic activity [66].

While controversial and possibly dependent on experimental setup, H_2S seems to modulate iNOS expression and function, indicating that H_2S targets ROS and RNS generation. H_2S suppressed iNOS upregulation in a rat diabetes mellitus model [67] and a novel, slow-releasing H_2S donor inhibited LPS-induced iNOS expression in macrophages [58•]. In contrast, others reported H_2S-mediated augmentation of iNOS expression in kidney proximal tubular epithelial cells [62]. iNOS regulation appears to be an important regulatory function of H_2S, albeit the exact mechanism remains still undetermined.

H_2S can also regulate cellular responses through the Keap1/Nrf2 regulatory pathway. In steady-state conditions Nrf2 is constantly degraded via the ubiquitin-proteasome pathway in a Keap1-dependent manner, while in the presence of ROS or electrophiles Nrf2 is stabilized, translocates to the nucleus, heterodimerizes with MAF proteins and regulates target gene expression via antioxidant response elements (ARE). In a murine model of diabetes mellitus, H_2S-induced *S*-sulfhydration of Keap1 promoted disulfide bond formation and subsequent release of Nrf2 [68,69]. Nrf2 accumulated in the nucleus, regulating the expression of HO-1 [69]. Using a mycoplasma infection model, H_2S again upregulated the expression of ARE-regulated genes, specifically HO-1, Prdx, and SOD1 [70]. In other studies H_2S increased SOD1 activity *in vitro* [65] and underwent H_2O_2-mediated, SOD-catalyzed oxidation to form persulfides [3]. Not only H_2S, but also H_2O_2 can enhance Nrf2 transcriptional activity by modifying Keap-1. Cardiomyocyte-restricted Nox4 overexpression in mice increased antioxidant gene expression including glutathione S-transferase α2, thioredoxin reductase 1, and NADPH dehydrogenase

quinone 1 [71], and this effect was abolished in Nrf2 null mice. In addition to effects on ARE-regulated genes, Nrf2 can directly suppress pro-inflammatory gene expression, in particular IL-1β and IL-6, via allosteric hindrance [72].

Pro-inflammatory effects have also been attributed to H_2S. Decreased H_2S production by inhibiting CSE pharmacologically in a rat model of severe acute pancreatitis led to a reduction in pro-inflammatory cytokines, specifically IL-1 and TNFα [73]. Others reported that enhanced synovial fluid H_2S levels correlated with increased clinical disease scores in inflammatory disease [74]. However, H_2S was also linked to protection from ischemia-reperfusion injury in rats [75]. H_2S signaling in inflammation and repair seems to be highly dependent on the overall context.

H_2S and per/polysulfides have *bone fide* immunological effects independently of other reactive species, and can modulate RNS and ROS levels and their signaling potential. Although many reports attribute protein sulfenation to H_2S directly, Mishanina *et al.* have recently questioned this assumption. In their view H_2S lacks the chemical reactivity required, while proposing persulfides and polysulfides as biological agents enacting sulfenation [76]. RSS research may currently lag behind that of ROS and RNS; however, the field promises varied and exciting research avenues.

Conclusions

The interplay between different types of reactive species contributes to the redox response of cells. It is important to differentiate and characterize the type, source and location of reactive species when investigating their functional relevance in terms of redox signaling in the immune system. First steps in this direction are rhodamine-based dyes for detecting HOCl or fluorescent protein-based redox sensors [77,78,79•]. These tools can be coupled with dyes and sensors for the detection of other species and with specific inhibitors targeting the enzymatic source, thereby opening new avenues to study the complex nature of redox signaling and to dissect the function of individual reactive species in a discrete signaling cascade. Animal models, organoids or multicellular 2D models will be needed to confirm the relevance of redox signals in complex settings, particularly in settings that include host–microbe interfaces such as mucosal barrier tissues.

Conflict of interest statement

Nothing declared.

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- • of special interest
- •• of outstanding interest

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