# THE ROLE OF REACTIVE OXYGEN SPECIES IN PROTECTIVE IMMUNITY

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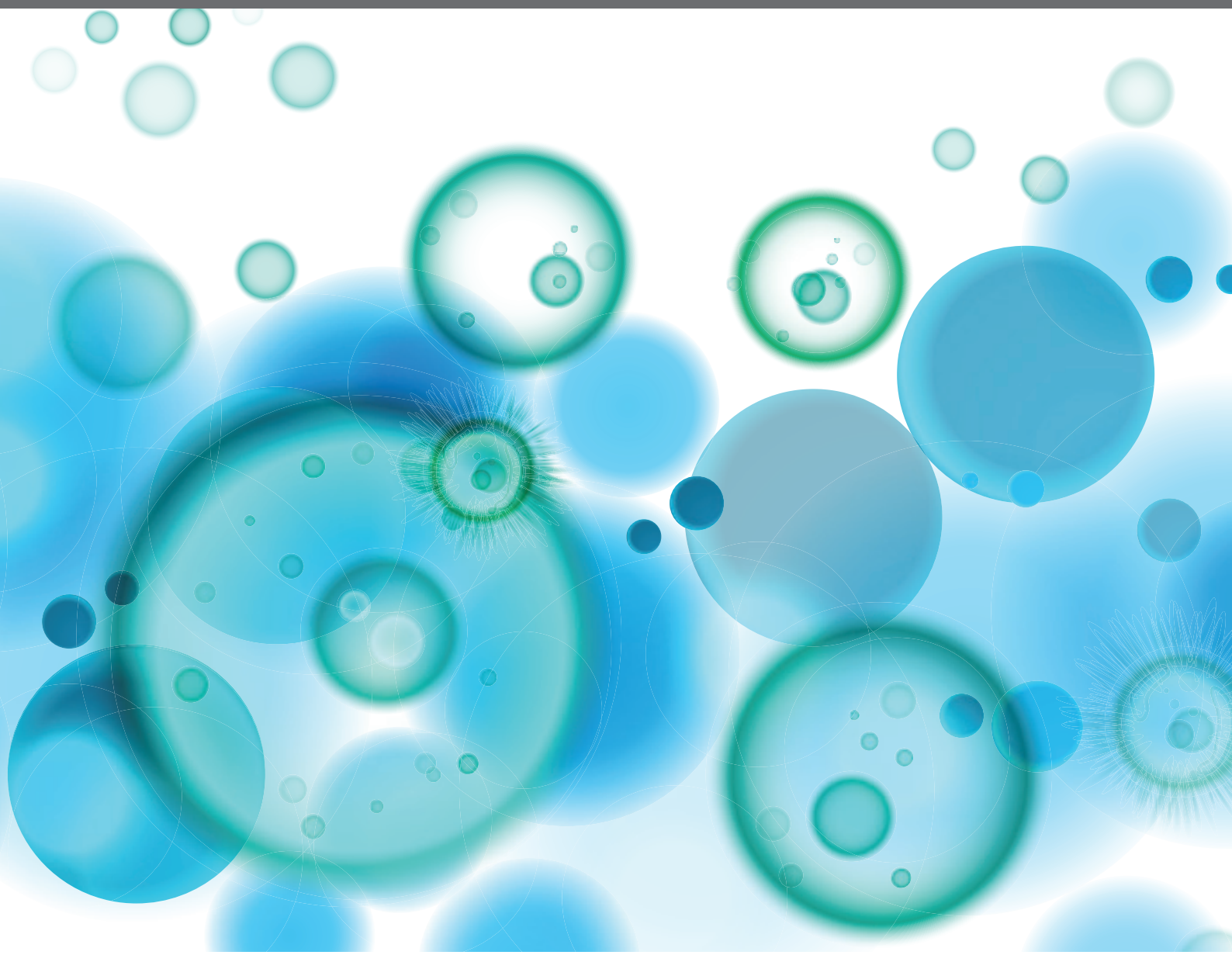

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# THE ROLE OF REACTIVE OXYGEN SPECIES IN PROTECTIVE IMMUNITY

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EDITORIAL
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# Editorial: The Role of Reactive Oxygen Species in Protective Immunity

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**Editorial on the Research Topic**

**The Role of Reactive Oxygen Species in Protective Immunity**

## INTRODUCTION

Reactive oxygen species (ROS) result from the partial reduction of oxygen, and encompass both radical [superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$), and nitric oxide (NO)] and non-radical [hydrogen peroxide ($H_2O_2$), hypochlorous acid (HOCl), and peroxynitrite ($ONOO^-$)] species having different half-life and reactivity. Classically, ROS have most often been considered harmful as they are common determinants of many cell death pathways including apoptosis, necrosis/necroptosis, ferroptosis, pyroptosis and autophagic cell death (1–8). This obvious ROS toxicity is certainly beneficial in oxidative killing of engulfed microbial pathogens in specialized immune cells, such as neutrophils (9) or macrophages (10). However, the simplistic view that ROS can only be harmful have been revisited. Indeed, ROS are essential to many physiological processes. ROS contribute to inflammation, vasoconstriction, signal transduction, cell migration, differentiation and proliferation (11–14). Moreover, $H_2O_2$ can modulate genes expression by redox based-epigenetic modification (15–17) and at the transcriptional level by activating redox responsive transcription factors such as AP-1, NRF2, CREB, HSF1, HIF-1, TP53, NF-κB, NOTCH, SP1, SCREB-1 and FOXO family (18–26). $H_2O_2$ also acts at the posttranscriptional level to control gene expression by regulating both cap-dependent and cap-independent translation (27–29). Because of their rather pleiotropic actions, ROS are critically important for cell biology, organ function, and system physiology including that of the immune system. In this Research Topic, we aimed at taking a close look at how ROS contribute to protective immunity.

## ROS IN ANTIMICROBIAL IMMUNE DEFENSE

ROS have been implicated in many aspects of the immune response to pathogens. ROS can damage biomolecules by oxidizing iron-sulfur clusters in a variety of enzymes leading to metabolic defects and release of iron. Free iron can react with hydrogen peroxide ($H_2O_2$) to give rise to aggressive hydroxyl radicals that can damage any biomolecule, including DNA. Therefore, ROS are essential for pathogens

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killing by phagocytic cells as illustrated by chronic granulomatous disease (CGD), an inherited disorder of the NADPH oxidase characterized by recurrent and severe bacterial and fungal infections (30). Reciprocally, microbes developed many strategies to counteract ROS- dependent host defense mechanism as discuss by Sen and Imlay. Indeed, microbes sense the $H_2O_2$ *via* OxyR or PerR transcription factors, or use the Grx3/Yap1 system to initiate enzymes that reduce cytoplasmic $H_2O_2$ concentrations, decrease the intracellular iron pools, and repair the $H_2O_2$-mediated damage and suit their particular environmental niches.

In addition to the ability of ROS to directly kill bacteria in the phagosome by oxidative damage to essential biomolecules (31), they can also trigger pathogen defense of phagocytes by various non-oxidative means, such as autophagy, receptor signaling, extracellular traps formation and instructing lymphocyte responses. Interestingly, ROS critically orchestrate the inflammatory process by regulating cytokine production during infection. Adding to this line of research, Hatinguais et al. demonstrated that sublethal ROS were essential to proinflammatory cytokine expression in the context of macrophages infected with swollen *Aspergillus fumigatus conidia*. The source of these ROS was identified in mitochondria by reverse electron flow, although NADPH oxidase 2 seemed to play a regulatory role in this proinflammatory pathway. A study by Buvelot et al. showed that sublethal doses of hydrogen peroxide trigger a coordinated and massive downregulation of genes involved in pyrimidine metabolism in *Staphylococcus aureus*, leading to reduced growth of extracellular bacteria and an increased sensitivity to added $H_2O_2$. Strikingly, as opposed to extracellular bacteria, intracellular pathogens were less affected, which could be a long-term survival strategy by allowing colonization through intracellular survival, while decreasing the risk of killing the host through dampened extracellular growth.

The induction of caspase-mediated host cell death can critically contribute to the elimination of intracellular pathogens *via* the destruction of their niche and *via* the induction of efferocytosis (31–33). However, many obligate intracellular pathogens have evolved mechanisms to inhibit programed cells death. Lavergne et al. discuss how this limitation is dealt with by the cytotoxic lymphocyte proteases, the granzymes. In addition to triggering host cell death, they also exert various non-cytolytic antimicrobial activities by directly degrading vital microbial proteins or hijacked host proteins crucial for the replication or survival of the pathogens. The granzymes also target microbial virulent factors. Interestingly, many mechanisms applied by the granzymes in this context rely on the induction of reactive oxygen species, either by promoting host cell apoptosis or by inhibition of pathogen growth. Whether the ROS involved in pathogen killing, cell death induction and the regulation of host cell metabolism originated from the same source is not fully elucidated and will need further study.

## REGULATION OF IMMUNE CELLS BY ROS

Beside the well-established ROS involvement in pathogen elimination, the wide range of the regulatory capacity of these reactive molecules is more and more revealed. Karmakar et al. discuss the regulatory mechanism downstream of the binding of Siglecs to sialic acid decorated receptor on immune cells. The Siglecs are a family of sialic-acid-binding immunoglobulin-like lectins believed to promote intercellular interactions and regulate the functions of innate and adaptive immune cells (34). Siglecs modulate immune activation and can promote or inhibit ROS generation under different contexts. dssSiglecs can bind sialoglycans present on the same cell (cis-interactions) or extracellular ligands present on neighboring cells or secretory glycoproteins (trans-interactions). Siglec-sialoglycan binding is weak and transient (22). The interaction between Siglecs with multivalent ligands leads to Siglec clustering, which increases the strength of Siglec-ligand binding and initiates cellular signaling.

ROS have also been proposed to be the common determinant of the inflammasome activation critical in the inflammatory process which is the determinant for an efficient immune response (35–38). The RNA-binding protein tristetraprolin (TTP) is an anti-inflammatory factor that prompts the mRNA decay of target mRNAs and is involved in inflammatory diseases such as rheumatoid arthritis (RA). lv et al. have shown that protein phosphatase 2A (PP2A)-mediated dephosphorylation regulates TTP to activate its mRNA-degrading function. Interfering with TTP expression or agonist of PP2A modulate monosodium urate (MSU) crystal-induced the expression of inflammation-related genes and NLRP3 inflammasome activation in a mitochondrial ROS-dependent manner suggesting that targeting TTP expression or function may provide a potential therapeutic strategy for inflammation caused by MSU crystals.

These studies demonstrate that ROS exert highly pleotropic functions in immunity. Therefore, it is essential to identify the specific intracellular sources of ROS and how they influence cellular processes in both physiological and pathological means, and how they impact on metabolic processes and inflammatory signaling as discussed by Canton et al. in the context of macrophages. Moreover, ROS are also required for full activation of lymphocytes as well as for the regulation of autoimmunity. Indeed, Bassoy et al. highlight the contribution of ROS in lymphocyte biology and stress their contribution in adaptive immunity with direct impact on the outcome of the antitumoral immune response as a consequence of the redox state in the tumor microenvironment (TME). Cali et al. showed the detrimental tolerogenic role of tumor-infiltrating myeloid cells (TIMs) actively dismantling effective immunity against cancer. TIMs inhibit T cell functions and promote tumor progression by multiple mechanism including the potentiation of the oxidative/nitrosative stress within the TME. They demonstrated that nitrosylation of granulocyte monocyte stimulating factor (GM-CSF) nourishes the expansion of this highly immunosuppressive myeloid subsets in tumor-bearing hosts.

The contribution of the ROS in adaptive immunity was further developed by Mortimer et al. who focus on the emerging role of NOX2-derived ROS in the development and maintenance of adaptive immunity and the effects of excess ROS in systemic disease. To this regard, Chávez and Tse discussed the

impact of mitochondrial-derived ROS and immunometabolism reprogramming in autoreactive T cell differentiation. Dysfunctional mitochondria have been involved in oxidative stress associated with many T cell-mediated autoimmune diseases. This agrees with the ability of mitochondrial-derived ROS to also contributed to T cell fate and function. Therefore, targeted manipulation of glycolysis and mitochondrial derived ROS could contribute to the elimination of autoreactive T cells while promoting immunosuppressive CD4 T regulatory (Treg). This targeted manipulation would have the advantage of avoiding global immunosuppression and preserving physiological immune response.

Finally, given the essential role of ROS for basic physiological functions as well as their contribution to pathophysiological situation, their therapeutic manipulation becomes an attractive strategy, although a non-trivial one. Dumas and Knaus discuss essential consideration for effective Redox medicinal approaches.

## CONCLUSION

This special Research Topic covers the extremely wide span of ROS function in immune defense and regulation and, with that, also the urgent need for further study to exploit these pleotropic molecules for therapy approaches. ROS are not only essential for antimicrobial defense but also exert a variety of crucial regulatory functions, reaching from the modulation of transcriptional programs to mediating differentiation fate. On the other hand, oxidative stress can contribute to various pathologies, including neuronal degenerative diseases, autoimmunity as well as cancer. Therefore, the potential therapeutic intervention in ROS biology will need extensive temporal and spatial fine tuning.

## AUTHOR CONTRIBUTIONS

DM and MW wrote the manuscript. All authors contributed to the article and approved the submitted version.

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# Mitochondrial Reactive Oxygen Species Regulate Immune Responses of Macrophages to *Aspergillus fumigatus*

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Reactive Oxygen Species (ROS) are highly reactive molecules that can induce oxidative stress. For instance, the oxidative burst of immune cells is well known for its ability to inhibit the growth of invading pathogens. However, ROS also mediate redox signalling, which is important for the regulation of antimicrobial immunity. Here, we report a crucial role of mitochondrial ROS (mitoROS) in antifungal responses of macrophages. We show that mitoROS production rises in murine macrophages exposed to swollen conidia of the fungal pathogen *Aspergillus fumigatus* compared to untreated macrophages, or those treated with resting conidia. Furthermore, the exposure of macrophages to swollen conidia increases the activity of complex II of the respiratory chain and raises mitochondrial membrane potential. These alterations in mitochondria of infected macrophages suggest that mitoROS are produced *via* reverse electron transport (RET). Significantly, preventing mitoROS generation *via* RET by treatment with rotenone, or a suppressor of site IQ electron leak, S1QEL1.1, lowers the production of pro-inflammatory cytokines TNF-α and IL-1β in macrophages exposed to swollen conidia of *A. fumigatus*. Rotenone and S1QEL1.1 also reduces the fungicidal activity of macrophages against swollen conidia. Moreover, we have established that elevated recruitment of NADPH oxidase 2 (NOX2, also called gp91phox) to the phagosomal membrane occurs prior to the increase in mitoROS generation. Using macrophages from gp91phox$^{-/-}$ mice, we have further demonstrated that NOX2 is required to regulate cytokine secretion by RET-associated mitoROS in response to infection with swollen conidia. Taken together, these observations demonstrate the importance of RET-mediated mitoROS production in macrophages infected with *A. fumigatus*.

**Keywords: macrophages, reverse electron transport, reactive oxygen species, mitochondria, *Aspegillus fumigatus*, cytokines**

## INTRODUCTION

*Aspergillus fumigatus* is a ubiquitous fungus that causes a wide range of illnesses, from allergic reactions to life-threatening invasive aspergillosis (1). Primary immunodeficiency is one of the conditions that places individuals at risk of deadly invasive aspergillosis (2). For instance, patients with chronic granulomatous disease (CGD), who have mutations in genes encoding the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex, are highly susceptible to bacterial and fungal infections including invasive aspergillosis (3, 4). The lack of NADPH oxidase (NOX)-derived reactive oxygen species (ROS) in CGD phagocytes results in the defective oxidative killing of pathogens (3).

Besides being crucial for intracellular oxidative killing, ROS can also orchestrate inflammatory processes in response to microbial pathogens including fungi (5–7). Changes in ROS levels are often a consequence of metabolic remodelling in activated immune cells. Together these changes in cellular metabolism and redox homeostasis can initiate, as well as resolve, inflammatory responses (8–12). Indeed, a growing number of investigations have shown that tuning the production of specific inflammatory mediators, cytokines, correlates directly with metabolic re-programming and altered ROS production (9, 11).

There are numerous potential cellular sources of ROS, but the mitochondrial respiratory chain complex I is known to be one of the main contributors (13). ROS can be generated at complex I *via* a specific mechanism called reverse electron transport (RET). RET occurs when a significantly reduced coenzyme Q pool and a large pH gradient across the mitochondrial inner membrane drive electrons backwards through complex I resulting in elevated superoxide production (14). Therefore, two types of metabolic alterations can lead to RET. First, RET may occur when the coenzyme Q pool becomes over-reduced with electrons from respiratory chain complex II or glycerol-3-phosphate dehydrogenase (9, 11, 14, 15). Second, a reduction in ATP production by oxidative phosphorylation may promote the increased membrane potential needed to sustain RET (9, 14).

The process of mitochondrial ROS (mitoROS) generation *via* RET has been known for decades, but its physiological relevance has been shown only recently (15). A growing body of evidence indicates that RET occurs under physiological conditions and that it drives redox signalling in a variety of processes *in vivo*. Specific targets of RET-generated mitoROS remain unidentified, but may include redox-sensitive proteins that are modified (and thus inhibited or activated) by mitoROS. Nevertheless, the generation of mitoROS by RET has been shown to be crucial for survival under stress (16), oxygen sensing by the carotid body (17), and control of inflammation (9, 11). In addition, although high levels of ROS can promote aging by causing oxidative damage to cellular components, increasing mitoROS production specifically *via* RET extends *Drosophila* lifespan (18). Therefore, identifying the mechanisms that trigger mitoROS generation *via* RET and defining their targets will improve our understanding of physiological and pathophysiological signalling transduction, and may suggest new avenues for therapeutic manipulation.

Previously, it was shown that macrophages stimulated by the bacterial product lipopolysaccharide (LPS) shift their metabolism from oxidative phosphorylation towards glycolysis, which has also been associated with an increase in RET-induced mitoROS production. In turn, mitoROS promote stabilization of the transcription factor, hypoxia-inducible factor 1α, resulting in elevation of interleukin (IL)-1β expression (9). It was recently demonstrated that infection with *A. fumigatus* enhances glycolysis in macrophages, which is required for efficient innate immune responses (19). In this study, we have shown that mitoROS are produced *via* RET in macrophages infected with swollen *A. fumigatus* conidia and that this contributes to redox signalling necessary for cytokine secretion and fungal growth inhibition. In particular, we have found that blocking RET with a mitochondrial inhibitor, rotenone, or an antioxidant, S1QEL1.1, prevents tumor necrosis factor-α (TNF-α) and IL-1β secretion in murine macrophages exposed to swollen fungal conidia. Furthermore, both rotenone and S1QEL1.1 abolish the ability of macrophages to inhibit growth of swollen conidia, while having no effect on their ability to inactivate resting conidia. Moreover, we show that enhanced recruitment of NOX2 (gp91phox) to phagosomes occurs before the increase in mitoROS generation by infected macrophages. Using macrophages from gp91phox$^{-/-}$ mice, we have further established that NOX2 activity is essential for the regulation of cytokine secretion *via* RET-derived mitoROS. Overall, our work reveals a novel mechanism underlying the regulation of antifungal responses of macrophages against *Aspergillus* infection.

**Abbreviations:** MitoSOX, red mitochondrial superoxide indicator; mitoROS, mitochondrial ROS; RET, reverse electron transport; NADPH, nicotinamide adenine dinucleotide phosphate; NOX, NADPH oxidase; ELISA, enzyme-linked immunosorbent assay; gp91phox$^{-/-}$, NOX2 deficiency; ROS, reactive oxygen species; CGD, chronic granulomatous disease; LPS, lipopolysaccharide; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; CTCF, corrected total cell fluorescence; BMDM, bone marrow-derived macrophages; MOI, multiplicity of infection; GLRX1, glutaredoxin 1; SOD2, mitochondrial superoxide dismutase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; DHN, 1,8-dihydroxynaphthalene; CI, complex I; CII, complex II; CS, citrate synthase; NF-κB, nuclear factor-κB; DPI, diphenyleneiodonium chloride.

## MATERIALS AND METHODS

### Reagents

Red Mitochondrial Superoxide Indicator (MitoSOX) (M36008), Pierce Trifluoroacetic Acid (TFA) (28904), dithiothreitol (DTT) (R0862), zymosan (Z2849) were obtained from Thermo Fisher Scientific. RPMI 1640 Medium, GlutaMAX (61870036), heat inactivated Fetal Bovine Serum (HI-FBS) (10082147), HEPES (1156049), EDTA (15575020), Phosphate-Buffered Saline (PBS) (10010023), HBSS (14025-092) were purchased from Gibco. Triton X-100 (X100), diamide (D3648), Triethylammonium bicarbonate buffer (TEAB) (T7408), NaCl (S9888), iodoacetamide (I1149), SDS (L3771), Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) (C4706), urea (U5378), acetonitrile (ACN) (271004), HEPES (H4034), rotenone (R8875), mitoTEMPO (SML0737), S1QEL1.1 (SML1948),

S3QEL2 (SML1554) were purchased from Sigma-Aldrich. Thiopropyl Sepharose 6B resin (17042001) was obtained from GE Healthcare.

## Cells and Preparation of *A. fumigatus*

Murine bone marrow-derived macrophages (BMDMs) were obtained from C57BL/6J or gp91phox$^{-/-}$mice, as described previously (20). Cells were differentiated in RPMI 1640 Medium, GlutaMAX supplemented with 20% (v/v) L929 cell supernatant, 10% (v/v) HI-FBS, 100 µg/ml penicillin, 100 µg/ml streptomycin, and 10 mM HEPES. At least one night before treatment, BMDMs were transferred into RPMI 1640 Medium, GlutaMAX supplemented with 10% (v/v) HI-FBS (R10 medium). Alveolar macrophages were isolated from murine bronchoalveolar lavage fluid obtained by washing the airways with sterile PBS containing 5mM EDTA (21). Murine peritoneal macrophages were harvested 4 days after intraperitoneal injection of 3% (w/v) thioglycollate (BD Biosciences) by washing the intraperitoneal cavity with sterile PBS containing 5mM EDTA (20). Isolated cells were plated in multi-well TC culture plates in R10 medium and left to adhere for at least 4 h with a subsequent washing off non-adherent cells.

*A. fumigatus* clinical isolate CBS 144-89 (CEA10) was used as wild-type strain (22). To test a DHN melanin-deficient strain, Δ*alb1*(*pksP1*) was used (23). To prepare swollen conidia of *A. fumigatus*, conidia were first grown in Sabouraud-dextrose media for 3 h at 30 rpm to avoid clumping. Swollen conidia were harvested, washed and re-suspended in R10 medium. To fix swollen conidia, after swelling, they were treated with 10% (v/v) formalin for 1 h at 4°C, subsequently washed with PBS and re-suspended in R10 medium. To prepare heat-killed conidia, a suspension of conidia in water were incubated at 90°C for 30 min.

## Measurement of Mitochondrial Reactive Oxygen Species and NOX2 Localization

For fluorescence microscopy, 4x10$^4$ BMDMs in R10 medium were inoculated into µ-Slide 8-well coverslip (ibidi) and incubated at 37°C in 5% (v/v) $CO_2$. Cells were then infected with *A. fumigatus* or exposed to zymosan. 20 min before the end of incubation, cells were stained with 5 µM MitoSOX in HBSS, and then fixed with 10% (v/v) formalin. To evaluate NOX2 cellular localization, BMDMs were exposed to conidia, methanol fixed and stained with gp91-phox Alexa Fluor 647 antibody (Santa Cruz, sc-130543 AF647). Fluorescence was measured using a DeltaVision fluorescent microscope. Images were analyzed using ImageJ software and measuring the corrected total cell fluorescence (CTCF). CTCF was calculated according to the formula: CTCF = Integrated Density - (Area of selected cell x Mean fluorescence of background readings). Brightness and contrast adjustments are the same for all representative images.

## Measurement of Mitochondrial Membrane Potential

The mitochondrial transmembrane potential (ΔΨm) was assessed by the measurement of uptake of MitoView633 (Cambridge Bioscience, BT70055) fluorescent probe that was monitored fluorimetrically in a microplate reader (Tecan, SPARK1933) with a far-red channel.

## Cysteine Oxidation

BMDMs at a concentration of 2 × 10$^6$ cells/well in TC-treated six-well cell culture plates were challenged with swollen *A. fumigatus* conidia (MOI 5) for 2 h. After incubation, macrophages were washed with PBS and samples were pre-processed for an analysis of oxidative protein modifications as described previously (24). Briefly, proteins were precipitated with TCA, and protein pellets were dissolved in 300 µl lysis buffer containing 1% (w/v) SDS, 150 mM NaCl, 100 mM TEAB, 4 mM EDTA, 1 tablet of cOmplete Ultra Protease Inhibitor Cocktail (Roche), and 100 mM iodoacetamide (IAM). After excess IAM was removed with acetone, proteins were re-solubilized in 100 mM TEAB and reduced by the addition of 0.5 M TCEP (tris(2-carboxyethyl)phosphine). Reduced proteins were captured on a pre-conditioned Thiopropyl Sepharose 6B resin. After incubation on the resin, unbound proteins were washed away with the washing buffers in the following order: 8 M urea; 2 M NaCl; 80% (v/v) ACN and 0.1% (v/v) TFA; and 25 mM HEPES. To elute cysteine-containing proteins, resin was incubated with 25 mM $NH_4HCO_3$ buffer containing 20 mM DTT and centrifuged at 1,500g for 1 min. Fractions were analyzed by SDS-gel electrophoresis. Protein concentration in each sample was determined by Bradford assay (25). Equal amounts of proteins were loaded and separated on NuPAGE 4–12% Bis-Tris Gel (Invitrogen) and gels were stained by silver staining kit (Thermo Scientific, 24612). SeeBlue Plus2 (Invitrogen, LC5925) was used as a marker. Western blot analysis was performed to evaluate the level of thiol modifications in GAPDH. MagicMark XP (Invitrogen, LC5602) was used as marker for western blots.

## Mitochondrial Respiratory Chain Assays and Evaluation of Protein Expression Levels

BMDMs were plated at 2 × 10$^6$ cells/well in non-treated six-well cell culture plates, challenged with *A. fumigatus* at MOI of 5 and plates were subsequently spun at 400g for 2 min. After incubation for 2 h, cells were washed with PBS and put on ice. Cells were harvested and centrifuged at 1000 g for 10 min at 4°C and frozen in liquid nitrogen. After cells were quickly thawed at 37°C, samples were dissolved in PBS containing cOmplete Ultra Protease Inhibitor Cocktail (Roche) and homogenized with a 50-µl Hamilton syringe (Sigma, 58382) by taking up and expelling the suspension several times until it appeared as a homogeneous solution. Homogenate was kept on ice for the immediate measurement enzymatic activities. Enzymatic activity of respiratory chain complexes was performed as previously described with a spectrophotometer Spark plate reader (26). Cell homogenates were also used to evaluate the abundance of proteins in BMDMs treated with swollen conidia of *A. fumigatus.* Extracted proteins were analyzed by western blot with anti-GAPDH (Abcam, ab181602), anti-GLRX1 (R&DSystems, AF3119), anti-SOD2 (Abcam, ab13533) or NOX2 (Abcam, ab129068) antibodies.

## Cytokine and Killing Assays

Macrophages were plated at $4 \times 10^5$ cells/well in TC-treated 48-well cell culture plates and left to adhere overnight. Cells were subsequently incubated with swollen *A. fumigatus* conidia (MOI 5). Zymosan (MOI 5) was given where indicated. 2 μM diphenyleneiodonium chloride (DPI) was added for 1 h to inhibit NADPH oxidase complex. 2.5 μM rotenone or 12 μM FCCP were added 1 h before challenge. 0.5 mM MitoTEMPO, 5 μM S1QEL1.1, or 5 μM S3QEL2 were added to macrophages for the whole period of incubation with swollen conidia. Supernatants from macrophages were collected at 14 h (overnight) after stimulation. IL-1β (DY401) and TNF-α (Dy410) ELISA kits from R&DSystems were used according to the manufacturer's instructions. To assess killing capacity, macrophages were plated at $5 \times 10^4$ cells/well in TC-treated 96-well cell plates and incubated with conidia at MOI of 3 for resting conidia and MOI of 1 for swollen conidia. Following incubation for 4 hours, cells were lysed and the viability of remaining conidia was determined with resazurin assay (27).

## Statistical Analyses

RStudio was utilized to perform data analyses and visualization. The following packages were used: base (28), car (29), data.table (30), ggplot2 (31), ggpubr (32), rstatix (33), plyr (34), carData (35). To test whether obtained data were normally distributed the Shapiro-Wilk test was performed. Levene's test was used to assess homogeneity of variance. Statistical significance was evaluated either with the unpaired standard Student's or Welch's *t*-test or Mann Whitney U Test. The false discovery rate (FDR) adjustment of the p values was implemented using the Benjamini-Hochberg method (36). Multiple groups were compared either with the Kruskal-Wallis test or Welch's one-way ANOVA or one-way ANOVA, all followed by Tukey post-hoc tests. * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$.

# RESULTS

## Sensing Swollen *A. fumigatus* Conidia Induces MitoROS in Macrophages

Recently, it was proposed that mitoROS, produced specifically through RET, transform activated macrophages into a pro-inflammatory state (9). However, it was not known whether the recognition of fungal cells, or fungal cell wall components, induces the increased mitoROS production in macrophages. Therefore, our first objective was to test whether macrophages infected with the fungal pathogen *A. fumigatus* display increased mitoROS generation. Interestingly, using mitoSOX as a fluorescent probe for mitoROS, we observed that resting conidia of *A. fumigatus* did not impact mitoROS production by BMDMs after a short-term co-incubation of 2 h (**Figures 1A, B**). In contrast to resting conidia, infection with germinating (swollen) conidia of the fungus for the same period led to elevated mitoROS production in macrophages (**Figures 1A, B**). The mitochondrial inhibitor rotenone is known to prevent mitoROS generation during RET (13, 37, 38) (**Figure 1F**), and therefore we tested the effect of treating BMDMs with rotenone before exposure to swollen conidia. Pre-treatment with rotenone for 1 h significantly inhibited the increase in mitoROS normally observed following exposure of BMDMs to swollen *A. fumigatus* conidia (**Figures 1A, C**). This was consistent with our prediction that *A. fumigatus* triggers elevated mitoROS generation in macrophages *via* RET.

As rotenone is a metabolic inhibitor, and therefore its potential toxicity could conceivably have accounted for the observed reduction in mitoROS production in infected macrophages. However, the rotenone concentration used (2.5 μM) did not alter viability of macrophages (**Figure 1D**). Even a 4-fold increase in rotenone concentration did not affect metabolic activity of BMDMs after a 1 h exposure (**Figure 1D**). In addition, mitochondrial membrane potential was not affected by rotenone (**Figure 1E**), suggesting that a short-term pre-treatment of BMDMs with 2.5 μM rotenone did not interfere with a mitochondrial potential-dependent accumulation of probes such as MitoSOX. These observations are consistent with a previous report about the dose- and time-dependent toxicity of rotenone (39). Therefore, the impact of this short-term pre-treatment of BMDMs with 2.5 μM rotenone supports the idea that *A. fumigatus* activates RET-mediated mitoROS production.

RET is characterized by a considerably reduced coenzyme Q pool and a subsequent transfer of electrons from coenzyme Q to complex I, and the increased activity of mitochondrial complex II can contribute to the excessive reduction of the coenzyme Q pool (**Figure 1F**). Therefore, we hypothesized that the elevated activity of complex II could facilitate mitoROS production *via* RET in fungi-challenged macrophages. As predicted, we found that the activity of complex II increased when BMDMs were treated with swollen conidia of *A. fumigatus*, compared to unstimulated BMDMs (**Figure 1G**). Furthermore, the activity of complex II was not affected by resting conidia, which were consistently unable to induce elevated mitoROS levels (**Figures 1A, B**). These data support the idea that complex II drives mitoROS production *via* RET.

Mitochondrial complex I is the site for mitoROS production *via* RET (**Figure 1F**), and the activity of this complex can be decreased due to inhibition by mitoROS produced at that site. Thus, the reduced activity of this complex is another indicator of RET taking place (38). Interestingly, when we measured the activity of complex I in infected cells, we could only detect a slight reduction in the complex I activity that was not statistically significant in BMDMs exposed to swollen conidia (**Figure 1G**).

Increased mitochondrial membrane potential, which occurs due to a reduction in ATP production by oxidative phosphorylation, can contribute to and help to sustain RET. We used MitoView633 to monitor mitochondrial membrane potential in infected BMDMs, as the mitochondrial accumulation of MitoView633 and its subsequent fluorescence are dependent on the membrane potential. To prevent overgrowth of the fungus during overnight co-incubation with BMDMs, we used inactivated conidia in this experiment. Consistent with our previous results, the mitochondrial

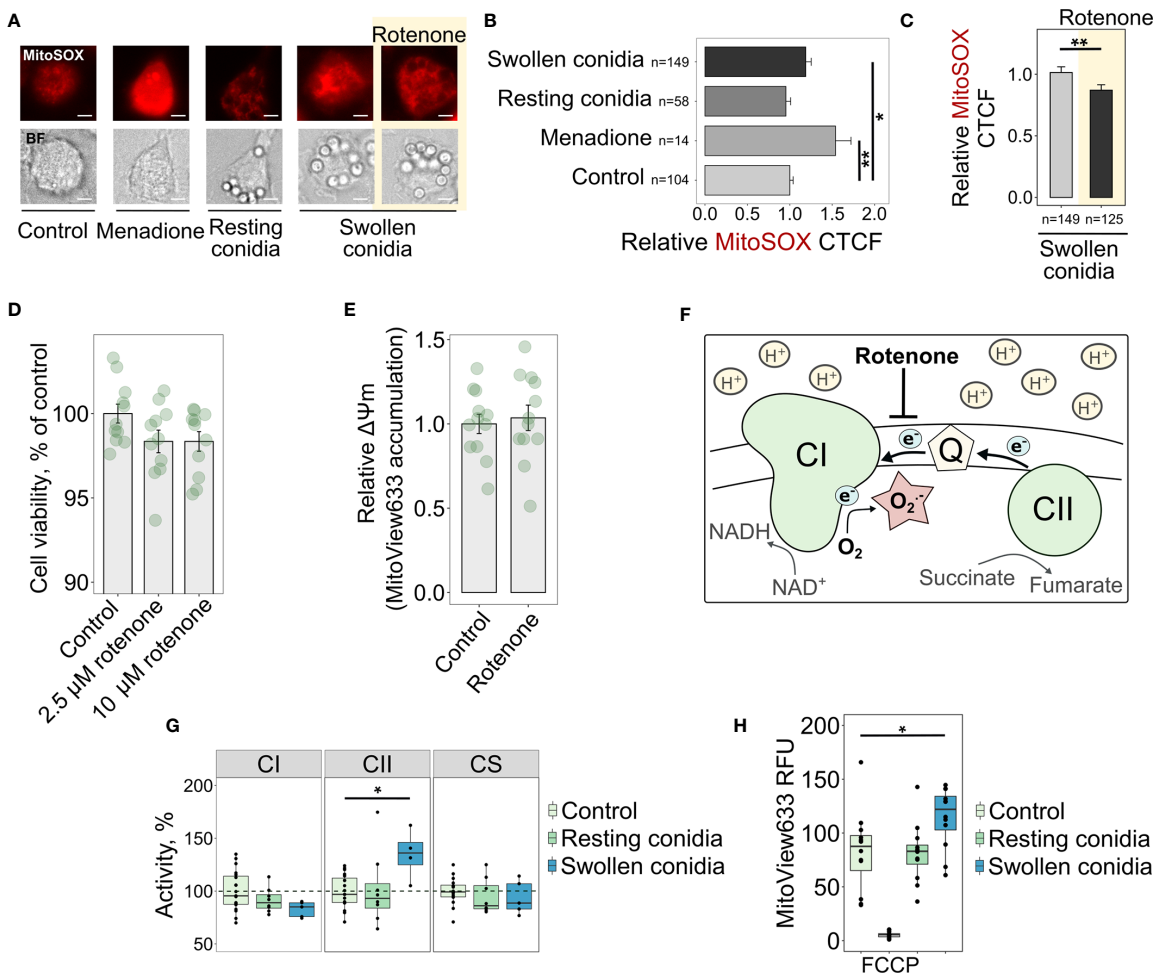


**FIGURE 1** | Sensing swollen conidia of *A. fumigatus* induces mitoROS production and increases the activity of the respiratory chain complex II and mitochondrial transmembrane potential. **(A)** Microscopic analysis of mitoROS in BMDMs infected with resting or swollen conidia of *A. fumigatus.* BMDMs were incubated with either resting or swollen *A. fumigatus* conidia for 2 h, stained with mitoSOX, fixed and subjected to imaging. Treatment with menadione was used as a positive control. To prevent mitoROS production through RET, BMDMs were treated with rotenone (shown with yellow background) for 1 h prior to exposure of BMDMs to swollen conidia. The scale bars are 5 µm. **(B, C)** The quantification of the level of fluorescence in infected macrophages. The abundance of mitoROS in BMDMs was determined by image analysis of MitoSOX-stained BMDMs with ImageJ software. A relative corrected total cell fluorescence (CTCF) was calculated in relation to an average of CTCF measured in untreated (Control) BMDMs **(B)** or in BMDMs exposed to swollen conidia **(C)**. **(D)** Measurement of viability of BMDMs after a short-term treatment with rotenone. Viability of BMDMs measured with the resazurin assay after 1 h of exposure to rotenone followed by 2 h cultivation in R10 media and is expressed in comparison to untreated cells (Control). **(E)** Analysis of mitochondrial membrane potential after treatment of BMDMs with rotenone. MitoView633-associated fluorescence was measured in BMDMs following 1 h of exposure to 2.5 µM rotenone followed by 2 h cultivation in R10 media containing fluorescent probe and represented mitochondrial membrane potential. Relative mitochondrial membrane potential was calculated in relation to an average of fluorescence units measured in untreated (Control) cells. **(F)** Schematic of mitoROS production *via* reverse electron transport (RET) in mitochondria. Coenzyme Q (Q) becomes over-reduced with electrons supplied by complex II (CII) during succinate oxidation. A large membrane potential and pH gradient drive electrons from coenzyme Q to complex I (CI) resulting in superoxide ($O_2^{\cdot-}$) production at one of CI sites. Rotenone inhibits RET-induced mitoROS generation. **(G)** Spectrophotometry analysis of enzyme activities of mitochondrial respiratory chain complex I (CI), complex II (CII), and citrate synthase (CS). BMDMs were left untreated (Control) or exposed to resting or swollen conidia of *A. fumigatus* for 2 h. Enzymatic activity measured in untreated BMDMs was set as 100% in each replicate. The activity of citrate synthase was measured to assess quality of mitochondria. **(H)** Measurements of mitochondrial membrane potential in BMDMs by detecting fluorescence of MitoView633. BMDMs were exposed to heat-inactivated resting or formalin-fixed swollen conidia in the presence of MitoView633. MitoView633-associated fluorescence was measured after incubation overnight. Treatment with 18 µM FCCP (p-trifluoromethoxyphenylhydrazone) was used as a negative control. Data are from two **(D, E, H)**, three **(B, C)**, or four **(G)** independent experiments. "n" indicates number of cells used for quantification; bars indicate means and standard errors. Statistical significance was calculated with the Kruskal-Wallis test followed by Tukey post-hoc tests **(B, H)**, with the Mann Whitney *U* Test **(C)**, or Student's **(D, E)** *t*-test, or one-way ANOVA followed by Tukey post-hoc tests **(G)**: * indicates $p<0.05$, and ** indicates $p<0.01$.

membrane potential was increased in BMDMs infected with swollen conidia, but not following exposure to resting conidia (**Figure 1H**).

Next, as *A. fumigatus* infection is associated with increased mitoROS (**Figures 1A–C**), we tested whether infection affects the redox status of endogenous BMDM proteins. To achieve this, we assessed the levels of reversibly oxidized proteins in macrophages infected with swollen conidia of *A. fumigatus*. Proteins were extracted from BMDMs and pre-processed according to the scheme in **Supplemental Figure 1A**. This method allowed us to capture proteins containing oxidized cysteines, which are the primary targets of ROS. This analysis of thiol oxidation showed that exposing macrophages to the fungus promoted oxidative post-translation modifications in the immune cells (**Supplementary Figure 1B**). We also tested whether the high mitoROS in fungus-activated BMDMs induces oxidative stress by evaluating the abundance of two antioxidant proteins: cytosolic glutaredoxin 1 (GLRX1) and mitochondrial superoxide dismutase (SOD2). The expression of neither protein was increased following exposure to swollen *A. fumigatus* conidia, as measured by western blotting (**Supplementary Figures 1C, D**) suggesting no significant exposure to oxidative stress.

## Removal of a Melanin or Protein Layer From Dormant *A. fumigatus* Conidia Does Not Induce MitoROS in Infected Macrophages

We speculated that DHN melanin on the surface of dormant *A. fumigatus* could prevent recognition of immunogenic components of the fungal surface required to initiate RET. Therefore, to investigate why resting conidia do not induce mitoROS *via* RET in macrophages, we exposed BMDMs to resting conidia of a *A. fumigatus* Δ*pksP* strain, which lacks the DHN melanin layer (23). Interestingly, we observed neither an increase in mitoROS levels (**Figures 2A, B**) nor a significant alteration in the activities of complex I or complex II, or in mitochondrial membrane potential in macrophages infected with this DHN melanin-deficient strain (**Figures 2C, D**).

Next, we tested whether proteins on the conidial surface could block the sensing of immunogenic fungal molecules that initiate mitoROS production *via* RET (40). Surface proteins were degraded by heating and heat-treated resting conidia were added to BMDMs. However, no changes in mitoROS production (**Figures 2A, B**) or in the mitochondrial activities and membrane potential were observed in macrophages following incubation with heat-killed resting conidia (**Figure 2C**, and **Figure 1H**). Overall, these data suggest that only germinating (swollen) *A. fumigatus* conidia are able to induce RET.

## Inhibition of RET in Macrophages Exposed to Swollen *A. fumigatus* Conidia Reduces Cytokine Secretion and Fungicidal Capacity

The secretion of pro-inflammatory cytokines such as TNF-α and IL-1β helps to develop host resistance to *A. fumigatus* infections and mediate antifungal immune responses (41, 42). Therefore, we tested whether blocking RET-mediated mitoROS production affects the ability of macrophages to secrete these cytokines in response to fungal invasion. Murine macrophages were pre-treated with rotenone for 1 h and exposed to swollen *A. fumigatus* conidia. We confirmed the importance of mitoROS for antifungal responses in bone marrow derived, alveolar, and peritoneal macrophages, as blocking mitoROS with rotenone resulted in reduced secretion of TNF-α and IL-1β by all three types of macrophages infected with swollen conidia (**Figures 3A, B**). This suggests a universal function of RET-associated mitoROS signalling in the antifungal responses of macrophages.

To confirm that the effect of rotenone is primarily due to its ability to suppress mitoROS production *via* RET, we tested whether other inhibitors of RET affect cytokine secretion in *A. fumigatus*-stimulated BMDMs. Accordingly, carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), which is known to prevent RET by altering mitochondrial membrane potential (14), significantly reduced cytokine secretion in infected BMDMs (**Figures 3C, D**). Also, S1QEL1.1, an antioxidant that suppresses mitoROS *via* RET (43), decreased TNF-α and IL-1β production by BMDMs exposed to swollen conidia. In contrast, other mitochondrial antioxidants, S3QEL2 and mitoTEMPO, which act independently of RET suppression (44), did not affect TNF-α secretion by BMDMs exposed to swollen conidia (**Figures 3C, D**). Taken together, these observations strongly suggest that preventing RET-mediated mitoROS generation impairs the cytokine responses of macrophages.

Next, we tested whether mitoROS production is necessary for macrophages to inhibit *A. fumigatus* growth. Interestingly, compounds with potential to suppress RET, namely rotenone, FCCP and S1QEL1.1, reduced the capacity of macrophages to inhibit growth of swollen conidia (**Figure 3E**). In contrast, the antioxidants S3QEL2 and mitoTEMPO, which do not affect RET, did not alter the fungicidal function of BMDMs against swollen conidia (**Figure 3E**). Interestingly, the RET inhibitors did not attenuate the inhibitory capacity of macrophages against resting conidia (**Figure 3F**). This is entirely consistent with our observation that resting conidia do not induce RET-mediated mitoROS generation (**Figures 1A, B, G, H**). Therefore, mitoROS that originate specifically from RET mediate cytokine secretion and are required to inhibit growth of swollen conidia, but are dispensable for the fungicidal function against resting conidia.

## Sensing β-Glucan or Viability-Associated Molecules Does Not Induce MitoROS *via* RET

The recognition of microbial ligands by various receptors is required to initiate immune responses and antimicrobial signalling (45). Currently, little is known about the ligands that induce inflammatory responses through RET-associated mitoROS signalling. Since we only observed RET and elevated mitoROS generation in BMDMs infected with swollen conidia, we reasoned that RET might be triggered by β-glucan, a major carbohydrate of the cell wall of swollen *A. fumigatus* conidia. Interestingly, macrophages activated by zymosan particles, a fungal cell wall-derived product composed mainly of β-glucan,

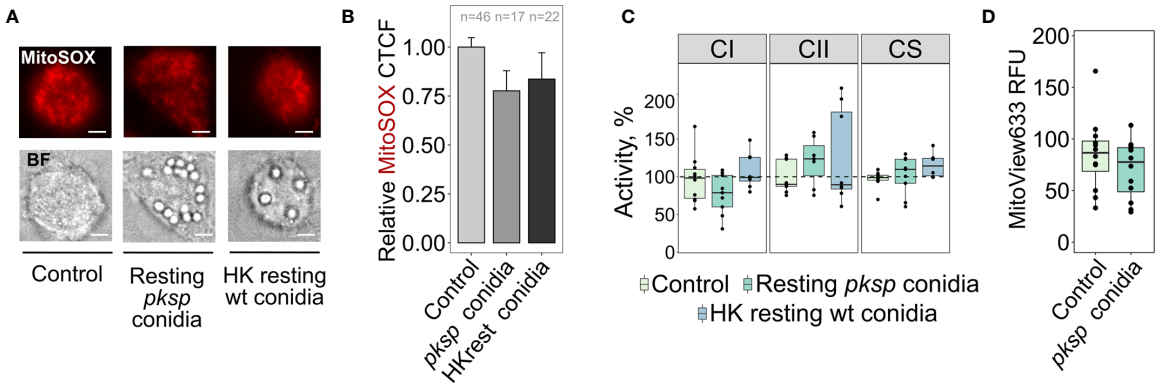


**FIGURE 2** | Detection of melanin-deficient or heat-killed resting *A. fumigatus* conidia by BMDMs does not induce mitoROS or alter the activity of mitochondrial complexes I and II, and mitochondrial transmembrane potential. **(A)** Microscopic analysis of mitoROS in BMDMs. BMDMs were left untreated (Control) or infected with conidia lacking DHN melanin (*pksp*) or heat-killed (HK) resting *A. fumigatus* conidia for 2 h. Cells were stained with mitoSOX, fixed and subjected to imaging. The scale bars are 5 μm. **(B)** The abundance of mitoROS in BMDMs was determined by image analysis of MitoSOX-stained BMDMs with ImageJ software. A relative corrected total cell fluorescence (CTCF) was calculated in relation to an average of CTCF measured in untreated (Control) BMDMs. **(C)** Spectrophotometry analysis of enzyme activities of mitochondrial respiratory chain complex I (CI), complex II (CII), and citrate synthase (CS). BMDMs were left untreated (Control) or exposed to resting or heat-killed resting conidia for 2 h. Enzymatic activity measured in untreated BMDMs was set as 100% in each replicate. The activity of citrate synthase was measured to assess quality of mitochondria. **(D)** Measurements of mitochondrial membrane potential in BMDMs by detecting fluorescence of MitoView633. BMDMs were exposed to *pksp* resting conidia. MitoView633 fluorescence was measured after incubation overnight. Data are from two **(B, D)**, or three **(C)** independent experiments. "n" indicates number of cells used for quantification; bars indicate means and standard errors. Differences between groups were evaluated with the one-way ANOVA.

exhibited elevated mitoROS generation as detected by mitoSOX staining (**Figures 4A, B**). However, the increase in mitoROS was not statistically significant and was not inhibited by rotenone (**Figures 4A–C**), suggesting that the mitoROS observed upon zymosan stimulation was not generated *via* RET. This correlated well with the observation that zymosan was unable to elevate complex II activity required for RET (**Figure 4E**) and that rotenone had no effect on TNF-α and IL-1β secretion when BMDMs were activated by zymosan (**Figures 4F, G**). Therefore, β-glucan recognition is not sufficient to trigger RET-mediated mitoROS production.

The metabolic reprogramming of macrophages, which could lead to mitoROS production *via* RET, takes place when immune cells are infected with live *E.coli*, whereas heat-killed bacteria do not have this effect upon macrophage metabolism (10). This may indicate that viability-associated molecules like microbial mRNA can induce metabolic reprogramming required for RET in immune cells (46). Thus, we also tested whether this "viability-specific" immune response triggers mitoROS in *A. fumigatus*-infected macrophages. Surprisingly, while heat-killed swollen conidia did not induce mitoROS, and even reduced their levels, infection with formalin-fixed swollen conidia led to an increase in mitoROS generation in BMDMs (**Figures 4A, B**). This elevation in mitoROS was abolished upon pre-treatment with rotenone, confirming that fixed germinating conidia can induce RET-associated mitoROS (**Figure 4D**). Furthermore, rotenone treatment reduced the production of TNF-α and IL-1β by macrophages challenged with fixed swollen conidia (**Figures 4F, G**). Meanwhile, heat-killed swollen conidia did not stimulate cytokine secretion by macrophages, which was consistent with their inability to initiate mitochondria redox signalling (**Figures 4F, G**). These data indicate that the regulation of cytokine production by RET does not depend upon sensing viability-associated molecules. Therefore, recognition of the cell surface of swollen conidia, is probably required for the induction of RET-mediated mitoROS production.

## NADPH Oxidase 2 Is Required for MitoROS Production *via* RET in *A. fumigatus*-Stimulated Macrophages

It was suggested previously that NADPH oxidases could affect mitochondrial function and particularly mitoROS production, and *vice versa* (47, 48). Also, the infection-associated metabolic reprogramming of macrophages requires the activity of the NOX2 complex (10). However, it is not known whether NOX2 (gp91phox) activity is required to initiate RET-associated mitoROS generation in activated immune cells. Therefore, we tested whether infecting macrophages with swollen conidia induces a simultaneous increase in NOX2 phagosomal accumulation and mitoROS production. Interestingly, during first hour of co-incubation of BMDMs with swollen conidia, there was enhanced recruitment of NOX2 to phagosomal membranes (**Figures 5A, B**). During this period mitoROS was not elevated in infected cells, but mitoROS generation did follow after 1.5 h of infection (**Figure 5C**). Therefore, NOX2 activation precedes enhanced mitoROS production. We then tested whether the increase in mitoROS is associated with changes in NOX2 abundance rather than its recruitment to the phagosome. However, both western blot and microscopic analyses revealed no detectable alterations in NOX2 protein abundance after 2 h of infection, during which time mitoROS levels were increased (**Figures 5D–F**). Therefore, enhanced NOX2 phagosomal

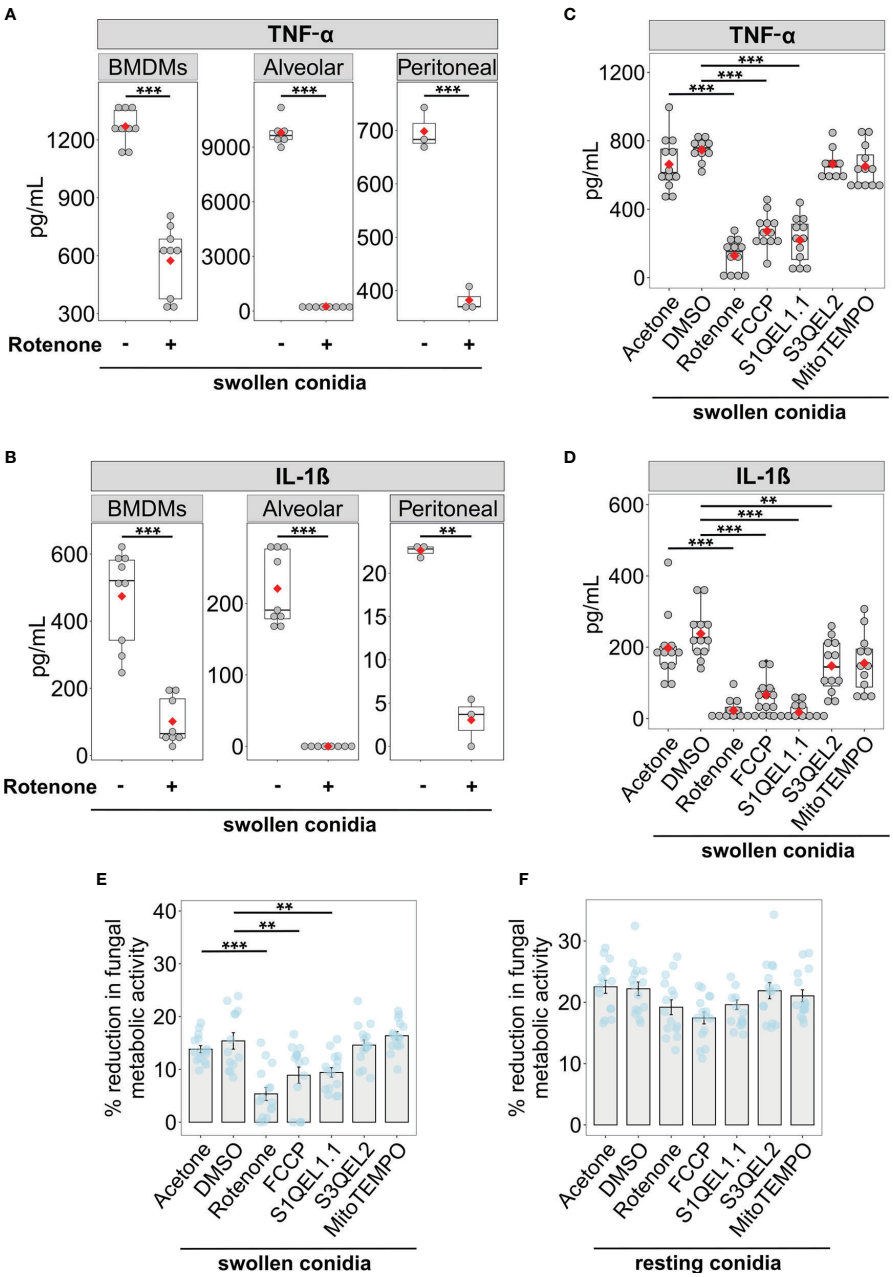


**FIGURE 3** | Suppression of RET-induced mitoROS reduces cytokine secretion and the ability to inhibit growth of swollen *A. fumigatus* conidia by murine macrophages. **(A, B)** Evaluation of cytokine secretion by infected macrophages. Bone marrow-derived macrophages (BMDMs), alveolar or peritoneal macrophages were treated either with vehicle (acetone) or rotenone for 1 h, after washing, macrophages were exposed to swollen *A. fumigatus* conidia and the supernatant was collected after incubation overnight. Levels of TNF-α **(A)** and IL-1β **(B)** in supernatants were analyzed by ELISA. **(C, D)** BMDMs were ether treated with vehicle (acetone or DMSO), rotenone, or FCCP for 1 h. After compounds were removed, BMDMs were treated with swollen *A. fumigatus* conidia. Antioxidants S1QEL1.1, S3QEL2, or mitoTEMPO were added together with conidia. Following incubation overnight, supernatants were collected to measure cytokine levels by ELISA. No cytokines were detected in the supernatant from cells treated with tested compounds, but not treated with swollen conidia (not shown). **(E, F)** BMDMs were ether treated with vehicle (acetone or DMSO), rotenone, or FCCP for 1 h. After compounds were removed, BMDMs were treated with swollen or resting *A. fumigatus* conidia. Antioxidants S1QEL1.1, S3QEL2, or mitoTEMPO were added together with conidia where indicated. After incubation for 4 h, macrophages were lysed with water containing Triton X-100, and the growth of *A. fumigatus* was measure by a metabolic activity assay based on resazurin. The metabolic activity of *A. fumigatus* conidia that were not exposed to BMDMs was set to 100%. Data are from two **(A, B)** (except for values for peritoneal macrophages, that were obtain from one experiment), or three **(C–F)** independent experiments. Bars indicate means and standard errors, red rhomb represent means. Statistical significance was calculated with the Welch's *t*-test **(A, B)**, one-way ANOVA **(C–E)** or Welch's one-way ANOVA **(F)** followed by Tukey post-hoc tests: ** indicates $p<0.01$, and *** indicates $p<0.001$.

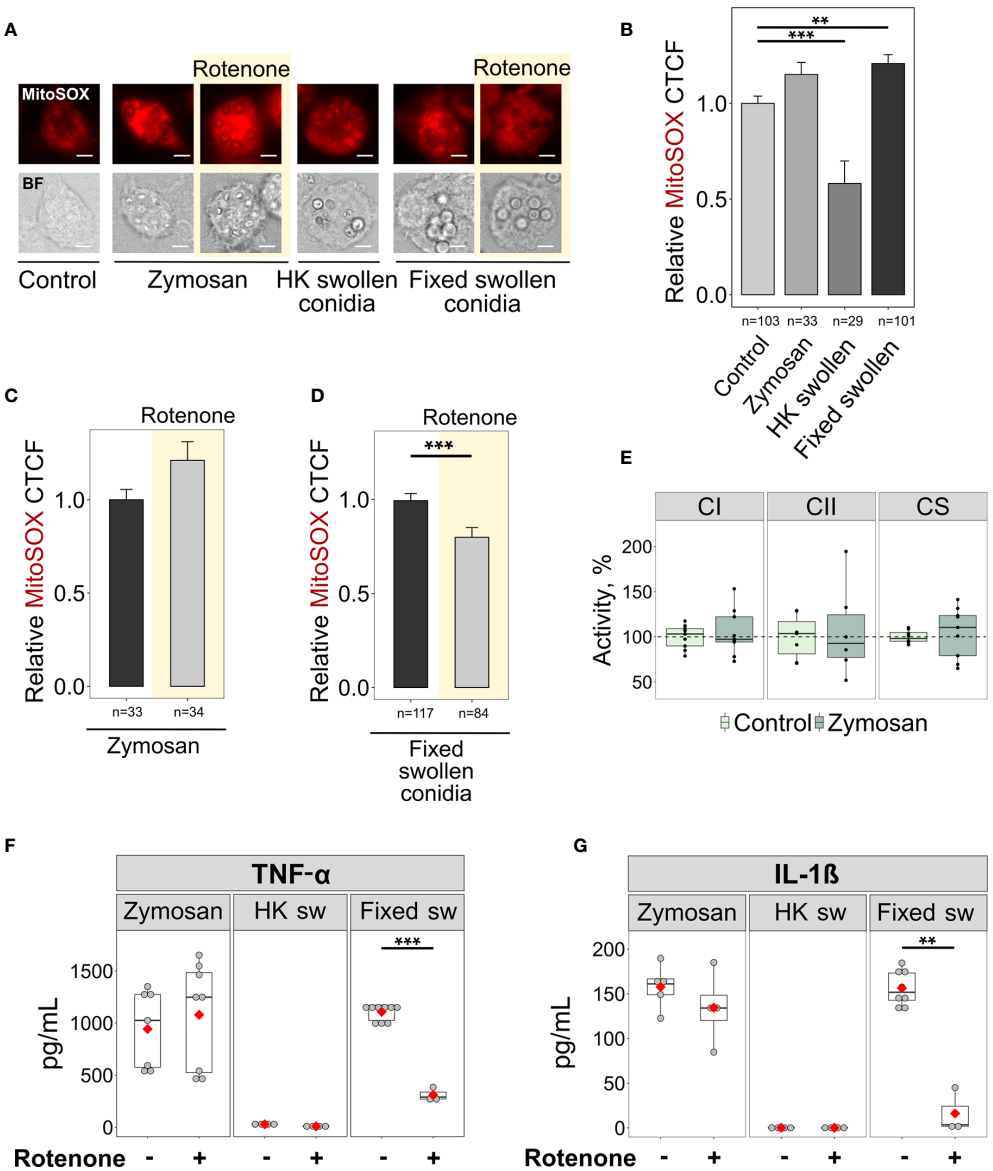


**FIGURE 4** | Sensing fixed swollen *A. fumigatus* conidia but not zymosan or heat-killed swollen conidia induces mitoROS production *via* RET in BMDMs. **(A)** Microscopic analysis of mitoROS in BMDMs. BMDMs were left untreated (Control) or exposed to zymosan particles, heat-killed (HK) or formalin-fixed swollen conidia of *A. fumigatus*. Cells were stained with mitoSOX, fixed and subjected to imaging. Where indicated, BMDMs were pre-treated with rotenone (shown with yellow background) for 1 h. The scale bars are 5 µm. **(B–D)** The abundance of mitoROS in BMDMs was determined by image analysis of MitoSOX-stained BMDMs with ImageJ software. A relative corrected total cell fluorescence (CTCF) was calculated in relation to an average of CTCF measured in untreated (Control) BMDMs **(B)** or in BMDMs exposed to zymosan **(C)** or formalin-fixed swollen conidia **(D)**. **(E)** Spectrophotometry analysis of enzyme activities of mitochondrial respiratory chain complex I (CI), complex II (CII), and citrate synthase (CS). Enzymatic activity measured in untreated BMDMs were set as 100% in each replicate. The activity of citrate synthase was measured to assess quality of mitochondria. **(F, G)** Evaluation of cytokine secretion in stimulated macrophages. After treatment with either vehicle or rotenone, BMDMs were exposed to zymosan particles, heat-killed (HK sw) or formalin-fixed (Fixed sw) swollen conidia of *A. fumigatus*. Levels of TNF-α and IL-1β in supernatants were analyzed by ELISA. Data are from two **(B–D, F, G)**, or three **(E)** independent experiments. "n" indicates number of cells used for quantification; bars indicate means and standard errors, red rhomb represent means. Statistical significance was calculated with the Kruskal-Wallis test followed by Tukey post-hoc test **(B)**, Mann Whitney U Test **(C, D)**, or Student's *t*-test **(E–G)**: ** indicates p<0.01, and *** indicates p<0.001.

recruitment precedes, and might be required for, the elevation of mitoROS generation. Interestingly, the induction of the elevated mitoROS generation were not depended on the uptake of swollen conidia as the majority of conidia were engulfed during the first 0.5 h of infection (**Figure 5G**).

Remarkably, NOX2 deficient BMDMs (gp91phox$^{-/-}$) exhibited higher basal levels of mitoROS in a resting state compared to wild-type BMDMs (**Figures 6A, B**). However, following challenge with swollen conidia, gp91phox$^{-/-}$ macrophages displayed no significant increase in mitoSOX

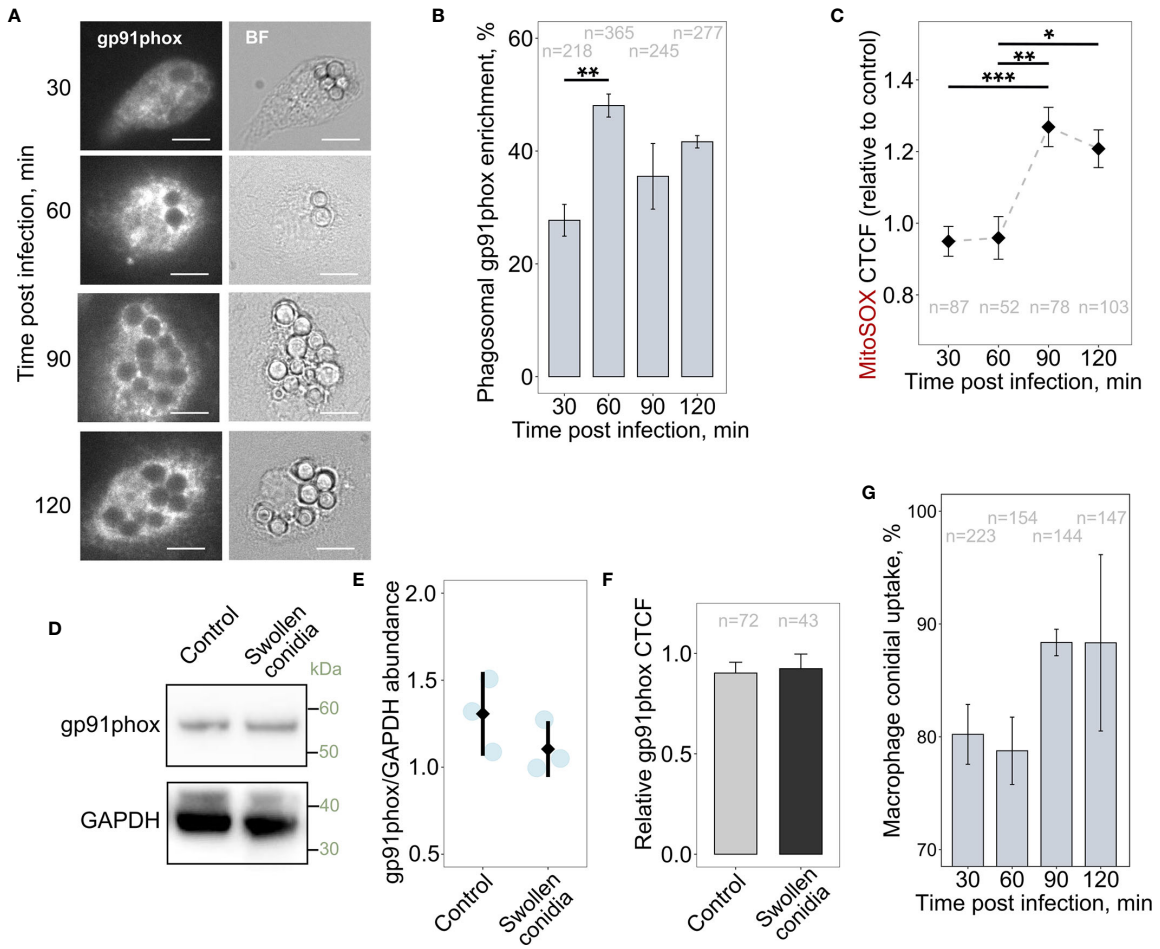


**FIGURE 5** | Recruitment of gp91phox to phagosomes occurs prior to the increase in mitoROS production. **(A)** Microscopic analysis of gp91phox localization in BMDMs. Cells were treated with swollen *A. fumigatus* conidia for an indicated period of time, fixed, stained with gp91phox antibodies, and subjected to imaging. The scale bars are 10 μm. **(B)** Quantification of the phagosomal enrichment of gp91phox in BMDMs following infection with swollen conidia. **(C)** The abundance of mitoROS in BMDMs was determined by image analysis of MitoSOX-stained BMDMs with ImageJ software. Corrected total cell fluorescence (CTCF) in infected cells was normalized to an average of CTCF measured in untreated (control) cells. Means of CTCF for each time point were compared. **(D)** Western blot analysis of protein abundance in untreated (Control) and infected for 2 h with swollen conidia BMDMs. Proteins were extracted from macrophages and analyzed by western blot with anti-gp91phox or GAPDH antibodies. **(E)** Quantification of intensity of corresponding bands on blots as shown on **(D)** from independent experiments. Images were analyzed in ImageJ. After background was subtracted, image was inverted and integrated density of each band was measured. Values were normalized to the integrated density from the corresponding lanes. Data presented in relation to a band intensity for GAPDH measured in each experiment. **(F)** Total cellular levels of gp91phox in untreated (Control) and exposed to swollen conidia for 2 h macrophages. A relative CTCF was calculated in relation to an average of CTCF measured in untreated (Control) BMDMs. **(G)** Uptake of swollen conidia by BMDMs was calculated by counting an average number of conidia per macrophage and was expressed as a percentage of the initial inoculum where MOI was set to 100%. Data are from two **(B, C, F)**, or three **(E, G)** independent experiments. "n" indicates number of phagosomes **(B)** or cells **(C, F, G)** used for quantification; bars indicate means and standard errors. Statistical significance was calculated with the one-way ANOVA **(B, G)**, Kruskal-Wallis test followed by Tukey post-hoc test **(C)**, or *t*-test **(E, F)**: * indicates $p<0.05$, ** indicates $p<0.01$, and *** indicates $p<0.001$.

cellular fluorescence (**Figures 6A–C**). Also, rotenone did not influence mitoROS levels in stimulated NOX2-deficient cells (**Figures 6A, B**). Accordingly, inhibition of RET with rotenone did not affect cytokine secretion in stimulated gp91phox$^{-/-}$ BMDMs, suggesting that RET did not occur in those cells (**Figures 6D, E**).

To examine the role of NADPH oxidase and associated mitoROS in inhibition of *A. fumigatus* growth by immune cells, we treated macrophages with diphenyleneiodonium (DPI), an NADPH oxidase inhibitor (49). First, we confirmed that as in NOX2-deficient cells, cytokine levels were not significantly affected by rotenone in DPI-treated BMDMs exposed to swollen conidia (**Supplementary Figure 2A**). Furthermore, the inhibitors of RET-mediated mitoROS, rotenone and S1QEL1.1, did not change the fungicidal activity of DPI-treated macrophages infected either with resting or swollen conidia (**Supplementary Figures 2B, C**). This provided additional evidence that RET did not take place in

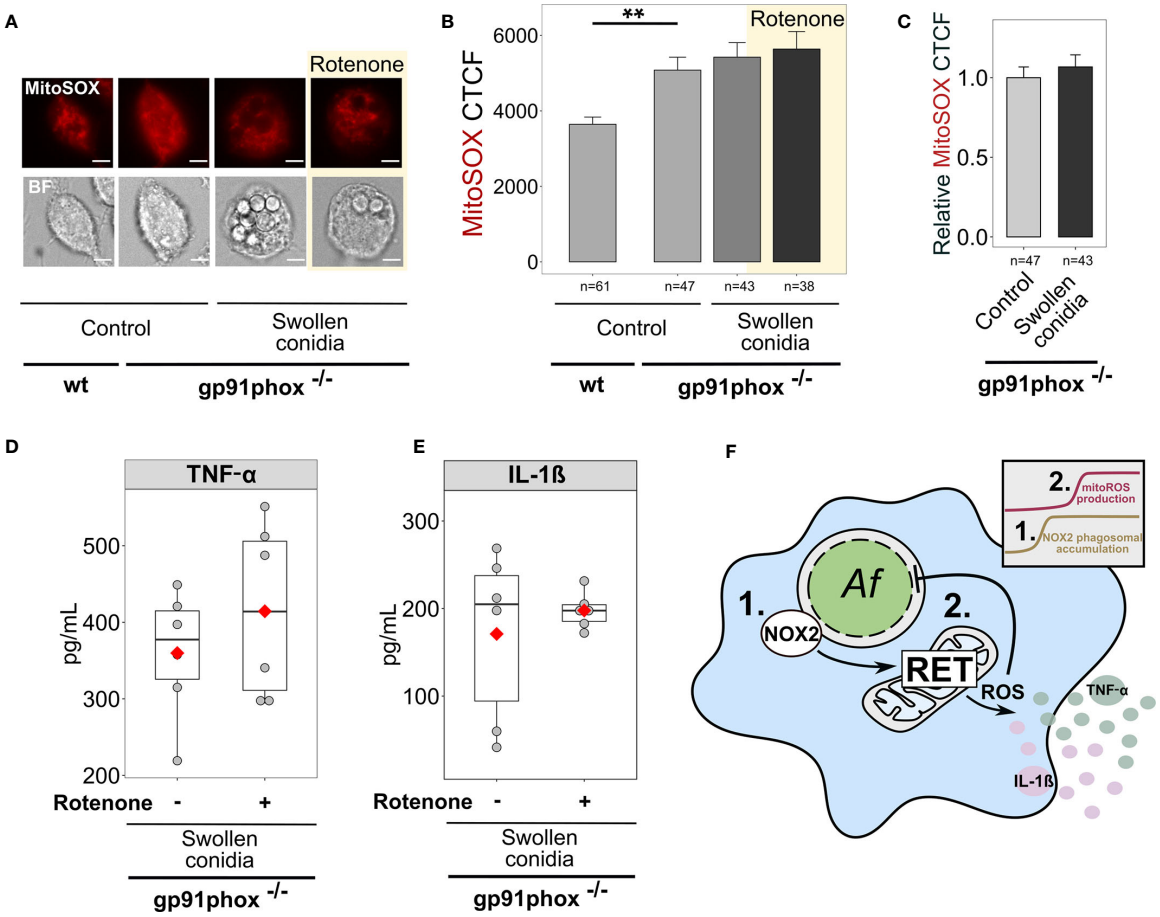


**FIGURE 6** | Regulation of cytokine secretion by mitoROS relies on the presence of NOX2 (gp91phox). **(A)** Microscopic analysis of mitoROS in BMDMs from wild-type (wt) or NOX2-deficient (gp91phox$^{-/-}$) mice. Cells were either left untreated (Control) or infected with swollen *A. fumigatus* conidia for 2 h, stained with mitoSOX, fixed and subjected to imaging. Where indicated, BMDMs were pre-treated with rotenone (shown with yellow background) for 1 h. The scale bars are 5 µm. **(B)** The abundance of mitoROS in BMDMs was determined by image analysis of MitoSOX-stained BMDMs with ImageJ software. Means of CTCF for each condition were compared. **(C)** MitoSOX CTCF was measured in untreated (Control) gp91phox$^{-/-}$ BMDMs and those exposed to swollen conidia for 2 h. A relative CTCF was calculated in relation to an average of CTCF measured in untreated (Control) gp91phox$^{-/-}$ BMDMs. **(D, E)** Evaluation of cytokine secretion by infected NOX2-deficient (gp91phox$^{-/-}$) BMDMs. Macrophages were infected with swollen conidia and supernatants were collected after incubation overnight. Levels of TNF-α and IL-1β in supernatants were analyzed by ELISA. No cytokines were detected in the supernatant from BMDMs (gp91phox$^{-/-}$) treated rotenone, but not treated with swollen conidia (not shown). **(F)** Schematic of the regulation of antifungal responses in macrophages through NOX2 complex and RET-associated mitoROS production. 1. Following infection with swollen conidia, gp91phox is recruited to the phagosomal membrane. 2. MitoROS production is increased due to RET. Activation of gp91phox is necessary for the regulation on cytokine secretion *via* RET-associated mitoROS. Data are from two independent experiments. "n" indicates number of cells used for quantification; bars indicate means and standard errors, red rhomb represent means. Statistical significance was calculated with the Kruskal-Wallis test followed by Tukey post-hoc test **(B)**, the Mann Whitney U Test **(C)**, the *t*-test **(D, E)**: ** indicates p<0.01.

infected cells with the defective NADPH oxidase. Also, the observed impact of FCCP, which disrupts mitochondrial membrane potential, suggested that mitochondria might be depolarized without a functional NADPH oxidase. Evidently, additional depolarization caused by FCCP negatively affected the fungicidal activity of BMDMs (**Supplementary Figures 2B, C**). To further examine the importance of NADPH oxidase and downstream mitoROS signalling *via* RET, we prolonged incubation of BMDMs with swollen conidia. Then, we discovered that after 6 h of infection, about 14% of fungal growth was inhibited by control macrophages, while only 7% of fungal growth was inhibited by DPI-treated macrophages, which was consistent with the previous report on alveolar macrophages (50). This suggested that while untreated macrophages could stop fungal proliferation, macrophages with the inhibited NADPH oxidase failed to prevent *A. fumigatus* growth. This confirmed the role of NADPH oxidase in sustained fungal growth inhibition by macrophages.

In summary, NOX2 defects prevent RET-associated mitoROS production and disrupt anti-*Aspergillus* functions of macrophages

mediated *via* this mechanism. This reveals a link between NOX2 and mitoROS production (**Figure 6F**) that is required for the regulation of antifungal responses in macrophages.

## DISCUSSION

This study addresses how mitoROS levels in macrophages orchestrate antimicrobial responses against the major fungal pathogen *A. fumigatus*. We found that increased mitoROS levels are triggered by germinating (swollen) conidia of *A. fumigatus*, but not by dormant (resting) conidia. RET contributes to these mitoROS levels, and this is related to elevated complex II activity in the respiratory chain and increased mitochondrial membrane potential. The elevation in mitoROS levels appears to rely on upstream NOX2-dependent signalling, ultimately leading to the enhanced production of pro-inflammatory cytokines that include TNF-α and IL-1β. Together, these results reveal that RET-generated mitoROS contribute to the antifungal responses of macrophages to *A. fumigatus*.

Coenzyme Q binding sites at complex I and complex III are among major producers of ROS in mitochondria (51). MitoROS generated at complex III was proposed to regulate cytokine secretion in *Listeria monocytogenes*–infected macrophages (52). Interestingly, in our study, a compound that specifically inhibits ROS production at coenzyme Q binding site of complex III, namely S3QEL2 (53), did not alter the ability of macrophages to inhibit *A. fumigatus* growth or secrete TNF-α. This suggested that complex III was not the main source of elevated mitoROS in fungus-stimulated macrophages.

It has been also proposed that enhanced mitoROS production *via* RET at complex I is crucial to transform macrophages into a pro-inflammatory state upon activation with LPS (9). The process involves metabolic alterations in the macrophage that include increased succinate oxidation by complex II, which could potentially drive mitoROS production *via* RET (9, 10). Here, we demonstrate that exposure to swollen *A. fumigatus* conidia leads to elevated complex II activity in the infected macrophages, and to mitoROS formation *via* RET. Interestingly, fungal viability is not essential to trigger RET, as formalin-fixed swollen conidia were sufficient to initiate mitoROS *via* this pathway. However, given that heat-killed swollen conidia fail to induce elevated mitoROS levels, heat-sensitive molecules on the fungal surface, or the heat-sensitive organization of these molecules on the surface, seem to trigger RET in the macrophage.

One of the phenotypic outcomes of enhanced mitoROS in stimulated macrophages is the modulation of cytokine release. The exact mechanism by which mitoROS affect cytokine secretion remains unclear. However, we have shown that macrophages infected with swollen *A. fumigatus* conidia exhibit increased thiol oxidation in endogenous proteins. This is consistent with the report that in *L. monocytogenes*-infected macrophages, mitoROS modify the redox status of Nuclear factor-κB (NF-κB) essential modulator, which regulates the extracellular signal-regulated protein kinases 1 and 2 and NF-κB pathways to promote the synthesis of pro-inflammatory molecules such as IL1-β, TNF-α and IL-6 (52). In addition to regulating redox-sensitive regulators of cytokine secretion, mitoROS may also affect pathways that supply reducing equivalents, such as NADPH. For instance, mitoROS can cause reversible oxidative modifications of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which would result in the diversion of glycolytic intermediates towards the pentose phosphate pathway to generate NADPH (54, 55). In addition to providing the reducing power that fuels antioxidant systems (56), NADPH is also used by NOX2 to generate antimicrobial phagosomal ROS. The potential involvement of mitoROS in regulating GAPDH activity, and hence the supply of reducing equivalents for NOX2, requires further investigation.

In addition to the potential impact of mitoROS on NOX2 functionality, NOX2 itself directly influences mitoROS production in activated macrophages. This work confirms there is crosstalk between NOX2 and mitochondria. In particular, using NOX2-deficient cells, we have shown that NOX2 activity is required to trigger mitoROS through RET. It is conceivable that, upon infection, NOX2-derived ROS cause oxidation and activation of the Fgr kinase leading to enhanced mitochondrial complex II activity, thereby creating the conditions for RET to take place (10, 57). Alternatively, the ROS produced by NOX2 may cause depolarization of mitochondrial membranes, matrix swelling and alkalinization, thereby leading to elevated mitoROS levels (58–60). Interestingly, the inhibition of NOX2 complex assembly in the phagosome membrane by DHN melanin on the conidial surface (61) could have accounted for the lack of mitoROS production by macrophages exposed to resting conidia. However, this notion is not supported by our observation that dormant Δ*pksp* conidia, which lack DHN melanin, were unable to induce RET in macrophages. Taken together, these findings indicate that NOX2 activation, in conjunction with the recognition of fungal cell surface components unique to swollen conidia, are required to initiate RET-mediated mitoROS redox signalling.

The lack of RET-mediated mitoROS in macrophages after exposure to resting conidia may explain their limited inflammatory responses and their ability to preserve immune homeostasis (62). Upon inhalation of *A. fumigatus* conidia, pulmonary immune cells maintain sterilizing immunity by rapid clearance of these resting conidia (63). The absence of mitoROS signalling may help to prevent exaggerated immune responses to dormant conidia that do not pose a significant threat. On the other hand, we observed an increase in RET-mediated mitoROS production following the sensing of a more dangerous form of the fungus - germinating conidia with the potential to cause invasive disease. Therefore, the regulation of mitoROS production *via* RET may be important for scaling immune responsiveness by activating immune responses to swollen *A. fumigatus* while preventing inflammation to dormant conidia.

As well as being important for initiating pro-inflammatory signals, RET in immune cells might be involved in limiting inflammation and preventing tissue damage after prolonged stimulation (11, 12). Thus, it is intriguing to speculate that in NOX2-deficient cells, the absence of RET might be responsible for dysregulated inflammatory responses. Indeed, patients with chronic granulomatous disease (CGD) whose immune cells lack functional NOX2, suffer from

prolonged inflammatory reactions that can lead to tissue damage (64, 65). Although RET-mediated mitoROS production does not occur in NOX2-deficient cells, and thus does not contribute into cytokine induction, our data indicate that redox homeostasis might be disrupted in these cells due to high mitoROS levels. This would be consistent with previous studies claiming that the lack of NOX2 results in mitochondrial redox imbalance and oxidative stress (66–68). In particular, in CGD patients, increased mitoROS levels are responsible for spontaneous neutrophil extracellular trap formation and linked to autoimmune disorders (67). Therefore, further investigations are required to address whether the activation of RET in immune cells with redox imbalance, such as gp91phox$^{-/-}$ cells, would help to modulate and normalize dysregulated inflammatory reactions. To support this possibility, triggering RET was previously shown to rescue pathogenesis associated with severe oxidative stress and even to increase *Drosophila* lifespan (18). Therefore, the modulation of mitoROS production specifically *via* RET might be beneficial in various pathological conditions.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and approved by UK Home Office (project license P79B6F297).

## AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.641495/full#supplementary-material

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REVIEW
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# How Microbes Defend Themselves From Incoming Hydrogen Peroxide

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Microbes rely upon iron as a cofactor for many enzymes in their central metabolic processes. The reactive oxygen species (ROS) superoxide and hydrogen peroxide react rapidly with iron, and inside cells they can generate both enzyme and DNA damage. ROS are formed in some bacterial habitats by abiotic processes. The vulnerability of bacteria to ROS is also apparently exploited by ROS-generating host defense systems and bacterial competitors. Phagocyte-derived $O_2^-$ can toxify captured bacteria by damaging unidentified biomolecules on the cell surface; it is unclear whether phagocytic $H_2O_2$, which can penetrate into the cell interior, also plays a role in suppressing bacterial invasion. Both pathogenic and free-living microbes activate defensive strategies to defend themselves against incoming $H_2O_2$. Most bacteria sense the $H_2O_2$ *via* OxyR or PerR transcription factors, whereas yeast uses the Grx3/Yap1 system. In general these regulators induce enzymes that reduce cytoplasmic $H_2O_2$ concentrations, decrease the intracellular iron pools, and repair the $H_2O_2$-mediated damage. However, individual organisms have tailored these transcription factors and their regulons to suit their particular environmental niches. Some bacteria even contain both OxyR and PerR, raising the question as to why they need both systems. In lab experiments these regulators can also respond to nitric oxide and disulfide stress, although it is unclear whether the responses are physiologically relevant. The next step is to extend these studies to natural environments, so that we can better understand the circumstances in which these systems act. In particular, it is important to probe the role they may play in enabling host infection by microbial pathogens.

**Keywords: reactive oxygen species, OxyR regulator, peroxide sensing repressor (PerR), Yap1p, nitric oxide**

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## THE THREAT POSED BY OXYGEN

Life evolved 3.8 billion years ago in an anoxic world. The biochemical pathways of these primordial organisms were based upon iron-cofactored enzymes, as this transition metal is adept at both redox and ligand-exchange processes. One billion years later, the appearance of photosystem II began the release of diatomic oxygen into the atmosphere. The oxygen levels in the atmosphere remained relatively low for another billion years, because photosynthetically generated $O_2$ was quickly consumed through its chemical reduction by environmental ferrous iron and sulfide. Only later, once these reductants had been largely titrated, did oxygen accumulate to higher concentrations (1). However, when it did, $O_2$ created an environment that was—and remains—incompatible with

extant organisms, which have inherited their iron-centric metabolic plans from their anoxic forbears.

In part the problem is that molecular oxygen oxidizes ferrous iron to insoluble ferric hydroxide precipitates, making it difficult for cells to acquire enough iron to charge their enzymes. That problem has been substantially ameliorated by the evolution of a variety of iron-import tactics (2). However, in addition, oxygen is kinetically active as a univalent oxidant (3). It disrupts the metabolism of anaerobic organisms by oxidizing their low-potential metal cofactors, thereby inactivating key enzymes, and by adducting the radical-based enzymes that play specialized roles in metabolism. Contemporary anaerobes have not solved this problem: In order to optimize their anaerobic growth, they continue to rely upon enzymes that are directly disrupted by oxygen, and so these microbes are constrained to anoxic niches (4).

In contrast, organisms that committed to life in oxic environments were able to dispense with low-potential catalytic strategies, and they employ enzymes that molecular oxygen does not damage at an important rate. Yet aerobes have residual problems with oxygen. Molecular oxygen is a passable univalent oxidant, and inside cells it adventitiously steals electrons from the cofactors of redox-active enzymes (5, 6). The transfer of a single electron results in the formation of superoxide (**Figure 1A**); the transfer of two electrons results in the formation of hydrogen peroxide. Both these species are more-potent univalent oxidants than molecular oxygen itself, and if left unchecked they can oxidize the exposed iron cofactors of enzymes that are found throughout metabolism. Further, a secondary reaction between hydrogen peroxide and cellular iron pools creates hydroxyl radicals (**Figure 1**), which are extremely potent species that can directly oxidize all cellular biomolecules (7). This vulnerability to partially reduced oxygen species (ROS) is universal among contemporary organisms. This review specifically aims to describe the strategies that are used by organisms to defray the toxicity of hydrogen peroxide—and to highlight the circumstances in which these defenses may not be adequate.

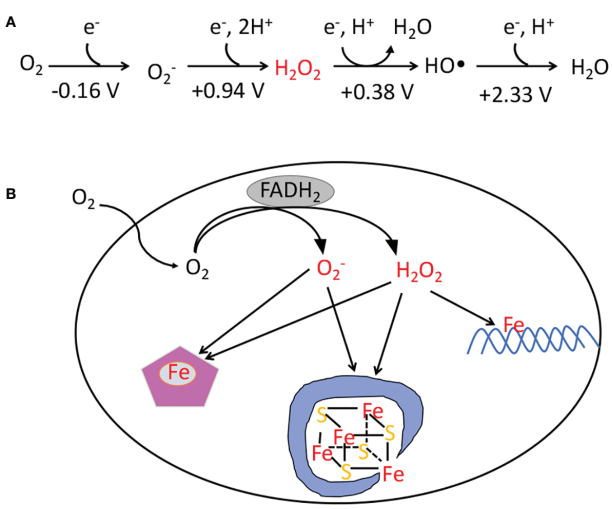


**FIGURE 1** | **(A)** The reduction potential of oxygen and reactive oxygen species. The standard reduction potentials (pH 7) indicate that unlike $O_2$, superoxide, hydrogen peroxide, and hydroxyl radicals are potent univalent oxidants. The standard concentration of oxygen is regarded as 1 M. **(B)** The classes of damage caused by intracellular $O_2^-$ and $H_2O_2$. The transfer of electrons from redox enzymes to oxygen generates superoxide and hydrogen peroxide. Both species can oxidize the solvent-exposed iron centers of mononuclear iron enzymes and [4Fe-4S] dehydratases. Additionally, $H_2O_2$ directly reacts with the intracellular iron pool, which is loosely associated with biomolecules, including DNA. The reaction generates hydroxyl radicals, which can damage DNA.

## REACTIVE OXYGEN SPECIES ARE CONTINUOUSLY FORMED INSIDE OXIC CELLS

The significance of hydrogen peroxide ($H_2O_2$) was first suggested by the discovery in 1900 of an enzyme devoted to degrading it: catalase (8). The discoverer, Oscar Loew, noted that it is found in virtually all tissues, and he made the inference that $H_2O_2$ was likely a by-product of metabolism that, if not removed, must be toxic to cells. Seventy years later Joe McCord and Irwin Fridovich chanced upon an enzyme that degrades superoxide ($O_2^-$) (9). Subsequent work has extended the cohort of scavenging enzymes to include peroxidases and superoxide reductases, and it has confirmed that virtually no organism lacks the ability to degrade $H_2O_2$ and $O_2^-$.

The model bacterium *Escherichia coli* contains two superoxide dismutases in its cytoplasm and one in its periplasm. Its cytoplasm also features both an NADH peroxidase (AhpCF) and two catalases (10) (**Figure 2**). Interestingly, some of these enzymes take advantage of the fact that iron can react with $O_2^-$ and $H_2O_2$: The original superoxide dismutase was likely an iron-dependent SOD, and most catalases use heme to degrade $H_2O_2$. Looking more broadly, similar scavenging systems are distributed through all biological kingdoms and in most cellular compartments, including the mitochondria, peroxisomes, and cytoplasm of eukarya.

The importance of these enzymes was revealed by genetic studies of *E. coli*: Mutants that lack cytoplasmic SODs, or that lack Ahp and catalases, were found to be unable to grow under oxic conditions in a standard glucose medium (10, 11). The observation confirmed Loew's postulate, proving both that these species are generated internally and that if not scavenged they will cripple cellular metabolism. Direct observation of $O_2^-$ production is not possible, but mutants that cannot degrade $H_2O_2$ release it into growth media at a rate that connotes an internal production rate of 10 μM/sec (12). Measurements show that as little as 0.5 μM intracellular $H_2O_2$ is sufficient to poison select biosynthetic pathways (13–15). The titers and kinetics of the scavenging enzymes are sufficient to suppress internal concentrations to about 50 nM—low enough to enable metabolism to operate without bottlenecks (12).

The orbital structure of $O_2$ restricts it to accepting a single electron at a time, and its reduction potential (- 0.16 V) is too

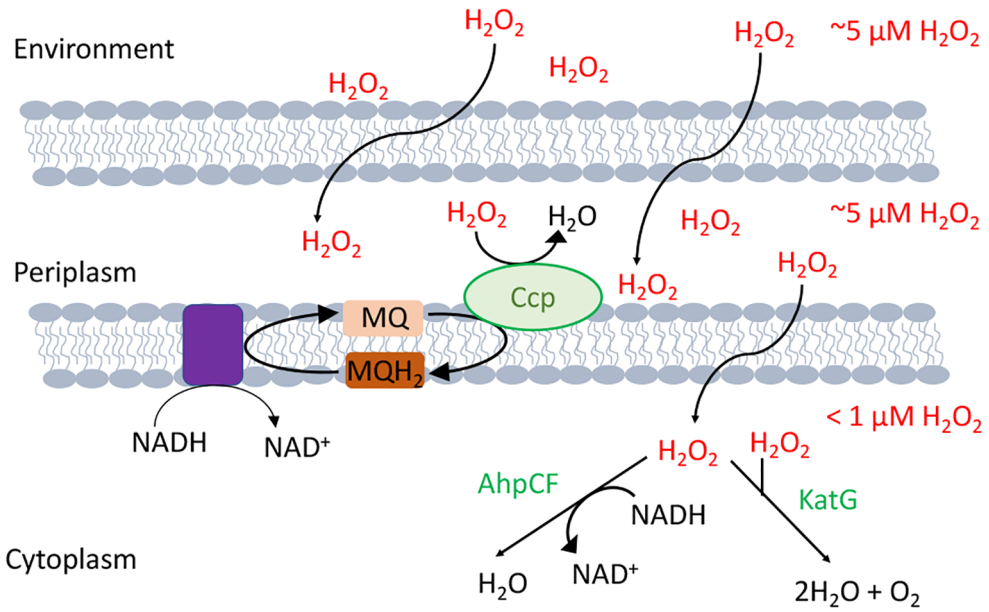


**FIGURE 2** | $H_2O_2$-scavenging enzymes in *E. coli*. Environmental $H_2O_2$ gradually diffuses into the cytoplasm, where it is degraded by NADH peroxidase (AhpCF) and catalase (KatG). Both are induced by OxyR. Cytoplasmic $H_2O_2$ is therefore substantially lower in concentration than is extracellular $H_2O_2$. Under hypoxic conditions OxyR also induces the periplasmic cytochrome c peroxidase (Ccp), which allows the respiratory chain to employ $H_2O_2$ as a terminal oxidant. Because $H_2O_2$ rapidly crosses through OM porins, and Ccp activity is moderate, the periplasmic $H_2O_2$ concentration is likely equivalent to that outside the cell.

modest for it to directly oxidize most biomolecules (3). However, $O_2$ can accept electrons from electron donors such as metal centers, flavins, and quinones. These are all prominent electron carriers in the *E. coli* respiratory chain, yet the rate at which cells produce endogenous $H_2O_2$ did not substantially diminish in mutants that lacked the respiratory enzymes, suggesting that in this bacterium $O_2^-$ and $H_2O_2$ are primarily produced by the accidental autooxidation of non-respiratory flavoproteins (5, 6) (**Figure 1**). These proteins are found throughout metabolism, and many, including glutathione reductase, lipoamide dehydrogenase, and glutamate synthase, have been shown to release ROS *in vitro* (16–18). The small size of $O_2$ prevents its exclusion from most active sites, and its collision with reduced flavins triggers the consecutive transfer of one or two electrons, generating $O_2^-$ or $H_2O_2$, respectively (19). The rate is naturally proportionate to collision frequency, meaning that ROS formation is more rapid in highly oxic environments. This math presumably underlies the observation that most organisms cannot tolerate oxygen levels that substantially exceed those of their natural habitat.

## THE CLASSES OF DAMAGE CAUSED BY SUPEROXIDE AND HYDROGEN PEROXIDE

The phenotypes of SOD mutants and of catalase/peroxidase mutants enabled investigators to track down the specific injuries that these ROS create. The mutants can grow at wild-type rates under anoxic conditions, but they require supplementation with aromatic and branched-chain amino acids if they are to grow in oxic media (11, 13–15, 20). They are also unable to use any carbon source, such as acetate, that required a fully functional TCA cycle (21, 22). Further scrutiny identified particular enzymes whose damage resulted in these defects.

The aromatic biosynthesis defect derives from the ability of $H_2O_2$ to oxidize the Fe(II) cofactor of the first enzyme of the pathway, DAHP synthase (15). The resulting Fe(III) atom dissociates. The role of the iron atom is both to bind substrate and to stabilize an oxyanion intermediate in the catalytic cycle; therefore, the resultant apoenzyme is completely inactive and the pathway fails. Other mononuclear Fe(II) enzymes such as ribulose-5-phosphate 3-epimerase, peptide deformylase, threonine dehydrogenase, and cytosine deaminase are similarly damaged (13, 14). Notably, the reaction between Fe(II) and $H_2O_2$ also generates a hydroxyl radical. If the catalytic Fe(II) atom is coordinated by a cysteine side chain, the hydroxyl radical reacts immediately with this sacrificial residue, creating a sulfenic acid (**Figure 3**) (13). Cellular thioredoxins and glutaredoxins can reduce this moiety back to a native cysteine residue, thereby allowing Fe(II) to bind and the activity to be restored (13). In contrast, when $H_2O_2$ oxidizes the Fe(II) of enzymes that lack such a residue, the nascent hydroxyl radical oxidizes other active-site ligands, creating lesions that are irreversible. These reactions are one source of the protein carbonylation that can be detected in $H_2O_2$-stressed cells (13).

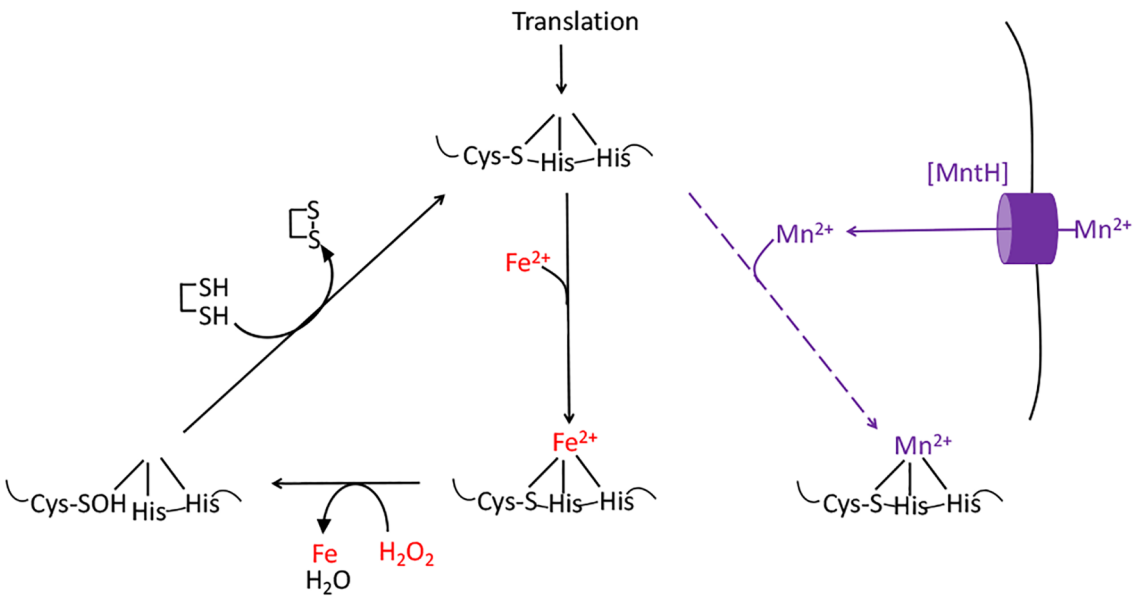


**FIGURE 3** | The damage caused to mononuclear iron proteins by hydrogen peroxide. $H_2O_2$ directly oxidizes the solvent-exposed Fe(II) cofactor, which then dissociates. The ferryl ($FeO^{2+}$) species that is formed in this reaction can directly oxidize the polypeptide ligands to the iron atom, irreversibly inactivating the enzyme. However, if a cysteine residue coordinates the iron, it will quench the ferryl radical (as shown). The enzyme activity can then be restored by reduction of the cysteine sulfenate residue, probably by thioredoxins. OxyR induces the MntH manganese importer, allowing the proteins to be metallated with Mn(II), which provides activity and does not react with $H_2O_2$.

The branched-chain biosynthetic defects as well as the TCA-cycle defects are caused by the oxidation of the [4Fe-4S] clusters of dehydratase enzymes (20, 22–26). A solvent-exposed iron atom of these clusters binds substrate directly, activating it for deprotonation and subsequently completing the dehydration by abstracting a hydroxide anion. But the same exposed iron atom can be oxidized by hydrogen peroxide to an unstable $[4Fe\text{-}4S]^{3+}$ state (**Figure 4**); this valence is unstable, and the cluster quickly disintegrates into a $[3Fe\text{-}4S]^{+}$ form that lacks the catalytic, solvent-exposed iron atom (24). Interestingly, the hydroxyl radical that is formed during this process pulls a second electron from the iron-sulfur cluster; thus, a hydroxide anion, rather than a hydroxyl radical, is released into the active site, and polypeptide oxidation is avoided. Cells continuously repair these damaged clusters, so that the steady-state activity of these enzymes reflects the balance between the oxidation and repair rates (21, 27, 28).

In addition to the metabolic defects described above, $H_2O_2$ can also react with the cytoplasmic pool of loose iron that is used to metallate nascent iron-dependent enzymes (**Figure 1B**). Iron is sticky, and this pool is thought to adhere to a wide variety of biomolecules, including the surface of nucleic acids (29). DNA thereby acts as a locus of hydroxyl radical production, and so DNA damage is a universal consequence of $H_2O_2$ stress (30–33). Some oxidative base lesions are mutagenic; others comprise replication blocks. All organisms therefore wield enzymes devoted to the excision or recombinational repair of oxidative lesions. Even though the level of endogenous $H_2O_2$ is well-controlled in scavenger-proficient *E. coli*, the rate of DNA oxidation remains high enough that mutants lacking these repair pathways cannot grow in oxic environments (34, 35).

Thus, organisms that dwell in oxic habitats can do so only because they have acquired an array of both scavenging and repair functions. The level of these enzymes is high and their synthesis is costly; accordingly, their titers have been calibrated to barely withstand the amount of stress commensurate with the oxygen level of the native environment (36, 37). This arrangement is successful under routine growth conditions. However, we shall see that it becomes inadequate if special circumstances elevate the production of ROS.

## THE PROBLEM OF EXOGENOUS OXIDATIVE STRESS

### Exogenous Sources of Superoxide

Both environmental photochemistry and chemical redox reactions generate $O_2^-$ (38), but the steady-state level of $O_2^-$ formed in this way is unlikely to be high enough to pose a risk for cells. Notably, the known targets of $O_2^-$ are iron enzymes that are cytoplasmic, and $O_2^-$ is a charged species that cannot cross membranes to get at them (39, 40). However, both microbes and higher organisms have evolved mechanisms by which they can use $O_2^-$ to poison unwanted competitors.

Mammals, plants, and amoebae have all weaponized an NADPH oxidase to kill bacteria (41, 42). Mammalian phagocytes engulf microbial invaders and spray them with

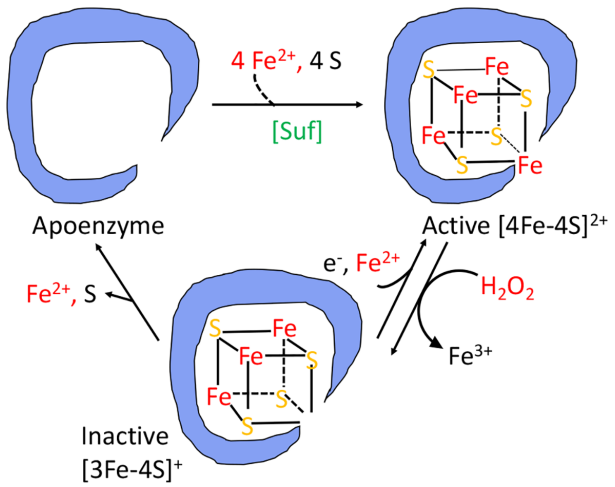


**FIGURE 4** | The damage caused to $[4Fe\text{-}4S]^{2+}$ cluster enzymes by hydrogen peroxide. The catalytic Fe atom of the dehydratase enzyme reacts with $H_2O_2$ and dissociates, leaving behind an inactive $[3Fe\text{-}4S]^{+}$ cluster. That cluster can be reactivated by reduction and remetallation. In some dehydratases the cluster completely disintegrates to form an apoenzyme. OxyR induces the Suf system to rebuild a functional holoenzyme.

superoxide that is formed by an inducible NADPH oxidase (**Figure 5**). The importance of this enzyme is reflected by the observation that humans and mice that lack it are vulnerable to infections (43, 44). However, it is still unclear how the phagocytic ROS production inhibits microbial growth. The acidic environment inside the phagosome can partially protonate $O_2^-$, resulting in a neutral species that in principle can penetrate captive bacteria; however, *Salmonella enterica* mutants lacking the periplasmic superoxide dismutase are hypersensitive, suggesting that $O_2^-$ does not gain access into the cytosol and instead acts on a target on the cell surface or in the periplasm (45, 46). Because protonated $HO_2$ is a better oxidant than $O_2^-$, it is possible that the acidity of the phagosome expands the range of biomolecules that superoxide can damage. The target has not yet been identified. A key difficulty is that *in vitro* systems have been unable to match the micromolar doses of superoxide (46, 47) that are sustained in the phagosome.

Bacteria also have found a way to impose $O_2^-$ stress upon competitors—and in this case the $O_2^-$ is aimed at the cytoplasm. A wide range of bacteria (and plants) secrete redox-cycling antibiotics (48–51). These are primarily soluble quinones and phenazines; they penetrate target cells, oxidize their redox enzymes, and transfer the electrons to oxygen. Enteric bacteria protect themselves from these compounds by activating the SoxRS regulon (52, 53). Its components elevate the titer of cytoplasmic SOD, pump out the drugs, and modify the cell envelope to diminish their entry (54).

Over the past dozen years microbiologists have examined the possibility that other bacterial stresses might also owe their potency, in part, to oxidative stress. The clinical antibiotics ampicillin, kanamycin, norfloxacin and trimethoprim have been particular foci of these studies, but similar hypotheses have been ventured for metal overload, nanoparticles, solvent stress, toxin/antitoxin systems, and many others (55). Key observations have been that the stressed cells accumulate oxidized forms of cell-penetrating dyes, which are thought to be oxidized by hydroxyl radicals; that cell death is slowed by the administration of thiol compounds, which might scavenge ROS, and by iron chelators that would block hydroxyl-radical formation; and that toxicity is diminished in mutants whose TCA cycle is blocked, ostensibly diminishing the rate of

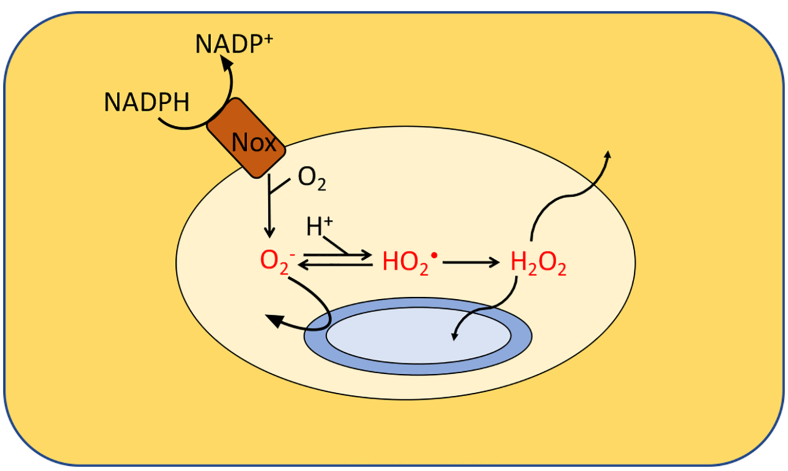


**FIGURE 5** | Formation of ROS by phagosomes. NADPH oxidase (Nox) generates superoxide which cannot penetrate the cytoplasmic membranes of the engulfed bacteria. It is believed that either superoxide or its protonated form injures extracytoplasmic targets. Additionally, membrane-permeable $H_2O_2$ is generated through dismutation. Calculations suggested that the levels of $O_2^-$, $HO_2\bullet$ and $H_2O_2$ in isolated macrophages range from 10–50 μM, 0.1–4 μM and 1–4 μM, respectively, depending upon phagosomal pH. Modeling predicts a similar $H_2O_2$ concentration inside neutrophils. The $H_2O_2$ levels would rise, however, if it accumulates it the surrounding tissue.

respiration and any associated ROS formation (56). However, this interpretation has been challenged (55, 57–59). Most of these protectants also have the capacity to slow metabolism, and it is well known that the efficacy of antibiotic action depends upon a robust growth rate. Moreover, tests of antibiotic action did not detect oxidative damage to ROS-sensitive enzymes or to DNA; the rate of $H_2O_2$ formation (measured in scavenging mutants) was not accelerated; and the *E. coli* response to $H_2O_2$ stress (below) was not triggered. Finally, no clear model has emerged to explain how such diverse stresses could create toxic levels of ROS. More work must be done to resolve these contradictory observations.

## Exogenous Sources of Hydrogen Peroxide

$H_2O_2$ is chemically formed in habitats though abiotic reactions between sulfur and oxygen at oxic/anoxic surfaces and the photochemical reduction of oxygen by chromophores (60–63). Levels can reach 1 μM in the ocean (64, 65). It can also be produced as part of the plant wound response, during the inflammatory response of mammalian hosts—and, notably, as a primary metabolic by-product of many lactic acid bacteria (66–70). The latter organisms often lack respiratory chains and use the two-electron reduction of molecular oxygen to recycle reduced NADH, thereby indirectly improving the ATP yield of what is otherwise a fermentative process. It seems likely that the excreted $H_2O_2$ may suppress the growth of competitors. The ability of lactic-acid bacteria to tolerate their own $H_2O_2$ is impressive: They can achieve high densities in lab cultures in which millimolar $H_2O_2$ has accumulated. This tolerance appears to be due to the absence of oxidant-sensitive dehydratases and mononuclear Fe(II) enzymes. This adaptation is not without a price: These bacteria are unable to synthesize many amino acids, and they lack a TCA cycle and the improved energy yield that comes with it.

Unlike superoxide, $H_2O_2$ is an uncharged, albeit polar, molecule that can cross cell membranes. Because this process is relatively slow, when bacteria venture into environments containing extracellular $H_2O_2$, the high activities of intracellular catalases and peroxidases succeed at lowering internal $H_2O_2$ concentrations below that of the external environment (12). The transmembrane gradient for *E. coli* has been estimated to be 5- to 10-fold between the external world and the cytoplasm. Indeed, although 0.5 μM internal $H_2O_2$ is sufficient to impair the growth of this bacterium in lab cultures, external concentrations of up to 5 μM seem to be tolerated without an overt growth defect (60). Thus the limited permeability of membranes to $H_2O_2$ is essential to the efficacy of scavenging enzymes and to the ability of bacteria to grow in many habitats.

Of great interest to biologists is the role that phagocyte-derived $H_2O_2$ may play in suppressing microbial infection (**Figure 5**). The $O_2^-$ that is produced by host NADPH oxidase will dismutate, either spontaneously or *via* enzymic catalysis, to generate $H_2O_2$. The ability of $H_2O_2$ to cross membranes likely enables it to enter phagocytosed bacteria—but it also allows it to diffuse across the phagosomal membrane, into the producing cell, and potentially out into extracellular environments. Modeling suggests that this effusion sharply limits the amount of $H_2O_2$ inside the macrophage phagosome, despite the rapid rate at which it is formed. Estimates are that the steady-state level falls well below 10 μM (46). Such doses may be enough to induce stress responses in the captive bacteria, but they are unlikely to be lethal. The major caveat to this analysis is that it presumes that the environment acts as a one-way sink for the $H_2O_2$. However, if $H_2O_2$ accumulates within inflamed tissue, the $H_2O_2$ flow is bidirectional, and the level that accumulates may in principle be far higher. Clearly, this question cries out for direct measurements of $H_2O_2$ *in vivo*.

The rate of $H_2O_2$ production in neutrophils is substantially higher than that in macrophages—but the fact that $H_2O_2$ is a substrate of myeloperoxidase has once again been projected to cap the level at which it can accumulate (47). In sum, although at first blush it seems a no-brainer that $H_2O_2$ would contribute mightily to the killing actions of these cells, that point is not yet resolved.

# THE ROLE OF OxyR DURING HYDROGEN PEROXIDE STRESS

When external levels of $H_2O_2$ exceed a few micromolar, its flux into microbes threatens to elevate its internal concentration to toxic levels, despite the action of scavenging enzymes. To cope, virtually all microbes possess inducible stress responses that are focused upon $H_2O_2$. The paradigmatic system is the OxyR response of *E. coli* (71). OxyR is a $H_2O_2$-activated transcription factor. It is not activated merely by movement of *E. coli* into oxic environments, and *oxyR* mutants are capable of normal aerobic growth. However, OxyR is activated when exogenous $H_2O_2$ accumulates to ~ 0.2 μM in the cytoplasm, which is achieved by about 3 μM external $H_2O_2$ (60, 72). Null mutants cannot grow at these levels of environmental $H_2O_2$.

The OxyR protein contains a sensory cysteine residue (C199) that is directly oxidized by $H_2O_2$, generating a sulfenic acid (-SOH) (73). As a result, the residue moves from the hydrophobic pocket in which it is buried and swings toward the C208 residue, which then condenses to form a disulfide bond (**Figure 6**). This bond locks OxyR into its activated conformer, and its DNA binding ability differs from that of the reduced protein. In *E. coli* the reduced form has little transcriptional impact upon most genes, but the oxidized form recruits RNA polymerase and thereby activates the expression of genes that possess an OxyR binding site. In many other bacteria, reduced OxyR acts as a repressor, and upon its oxidation it releases the DNA, stimulating gene expression (74, 75). In still other bacteria gene expression is both repressed by the reduced form and activated by the oxidized form. The conformational change is manifested by an alteration of its DNA footprint (76).

Free cysteine reacts very slowly with $H_2O_2$ (2 $M^{-1}$ $s^{-1}$), and typical cysteine residues of proteins do, too (77). Yet in OxyR the sensing cysteine residue reacts with a rate constant of $10^5$ allowing it to detect micromolar $H_2O_2$ in seconds (72, 78). In that respect the hyperreactive cysteine of OxyR resembles the catalytic cysteine residue of thiol-based peroxidases, including

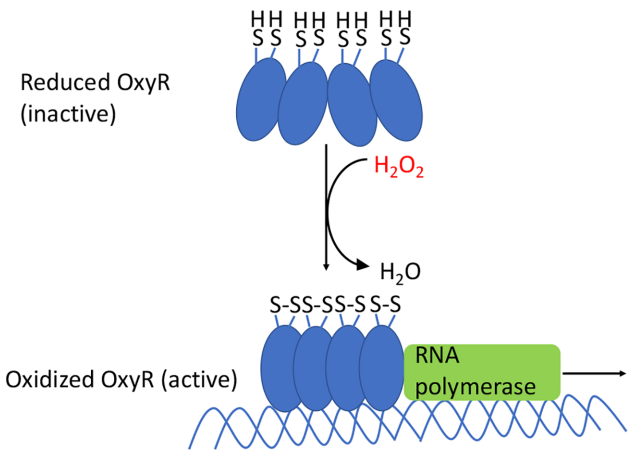


**FIGURE 6** | OxyR activation in *E. coli*. The oxidation of the sensory C199 cysteine by $H_2O_2$ leads to the formation of a disulfide bond between C199 and C208. The resulting conformational change causes OxyR to bind as a tetramer to the promoter regions, which recruits RNA polymerase, and results in the transcription of genes in the OxyR regulon. In many other bacteria the reduced form also binds DNA, albeit in an elongated conformation that represses transcription; oxidation again converts it to a transcriptional activator.

AhpC of *E. coli*. In the latter enzyme an adjacent cationic residue facilitates the deprotonation of cysteine, which provides an order-of-magnitude improvement in its reactivity with $H_2O_2$ (79). A plausible explanation for the remaining enhancement is that the nucleophilic cysteine is arranged in a large hydrophobic cleft. Surrounding hydrogen bonds could polarize the dioxygen bond, making it vulnerable to attack, and one of the residues can protonate the hydroxide leaving group, pulling the reaction forward. As a result, thiol-based peroxidases have a rate constant of $10^7 M^{-1}\ s^{-1}$, which is appropriate for their physiological role (80, 81). A similar physical arrangement may explain the high rate constant of OxyR as well.

It follows that the activation of OxyR is an excellent marker of $H_2O_2$ stress. This effect can be tracked by monitoring the expression of OxyR-controlled genes—or by visualizing the intrinsic fluorescence of HyPer, an engineered chimera of OxyR and yellow-fluorescent protein (82). HyPer is as responsive to $H_2O_2$ as is OxyR itself, and its oxidation status can be visualized either by microscopy or flow cytometry. Importantly, two-wavelength analysis can correct for variable HyPer content in different samples, thereby avoiding loading artifacts that can arise when redox-active dyes are employed as ROS sensors.

## THE DEFENSES THAT OxyR TURNS ON

When *E. coli* is stressed by an influx of $H_2O_2$, activated OxyR stimulates the transcription of over two dozen genes (83). These mainly fall into three categories: proteins that reduce the $H_2O_2$ concentration, proteins that shrink the iron pool, and proteins that deal with the damage that $H_2O_2$ produces (**Table 1**). The peroxidase AhpCF and the catalase KatG are each induced more than 10-fold in order to scavenge $H_2O_2$ (**Figure 2**). Why two enzymes? AhpCF is an efficient scavenger when the $H_2O_2$ concentration is less than 10 μM and the cell is well-fed (10). However, AhpCF requires NADH as a reductant, and so its activity becomes limited when catabolic substrates are scarce. In contrast, catalases do not require reductants, and they can degrade $H_2O_2$ faster than Ahp. However, catalases are problematic when the $H_2O_2$ concentrations are low, since their two-step catalytic cycle can stall with the heme in its intermediate ferryl radical form. This species is a potent oxidant, and unless it is quenched by a reductant, it can abstract electrons from the surrounding polypeptide and inactivate the enzyme (81). The KatG catalase of *E. coli*—and of many other bacteria—has a channel that apparently enables small-molecule reductants to approach the active site and quench the high-valence heme (84); for this reason the enzyme is denoted a catalase/peroxidase, though the peroxidase activity by itself is too slow to comprise an efficient scavenging mechanism (85).

When $H_2O_2$ levels are high, the existential threat to bacteria is that DNA oxidation will prove lethal. This damage is driven by the intracellular iron pool (86), and so the OxyR system employs several mechanisms to diminish it (**Figure 7**). Dps, a dodecameric mini-ferritin, is induced to sequester unincorporated iron in its hollow core (87–89). This action requires that loose Fe(II) be oxidized to Fe(III), and for this reaction Dps apparently uses $H_2O_2$ as a co-substrate. One benefit is that Dps will stop storing iron when $H_2O_2$ concentrations drop. The iron-uptake repressor Fur is also induced (90). In unstressed cells Fur:Fe(II) complexes signal that the cell has sufficient iron, and this form occludes the promoters of genes that encode iron import systems (2). When $H_2O_2$ is present,

**TABLE 1** | Genes induced by OxyR during hydrogen peroxide stress in *E. coli*.

| Gene | Function | Role during $H_2O_2$ stress |
|---|---|---|
| *ahpCF* | NADH peroxidase | Scavenge $H_2O_2$ |
| *katG* | Catalase | |
| *ccp* | Cytochrome c peroxidase | Uses $H_2O_2$ as a terminal electron acceptor |
| *dps* | Mini-ferritin | Reduce the intracellular iron pool |
| *fur* | Repressor of iron import | |
| *yaaA* | Unknown | |
| *clpSA* | Chaperone | Activate Fe/S enzymes |
| *sufA-E* | Iron sulfur-assembly | |
| *hemF* | Coproporphyrinogen III oxidase | Heme synthesis |
| *hemH* | Ferrochelatase | |
| *mntH* | Manganese importer | Activate mononuclear Fe enzymes |
| *gor* | Glutathione reductase | Maintain the thiol status |
| *trxC* | Thioredoxin | |
| *grxA* | Glutaredoxin 1 | |
| *oxyS* | Non-coding RNA | Role unknown |
| *isrC* | Non-coding RNA | |
| *flu* | Antigen 43 | |
| *fhuF* | Ferric iron reductase | |

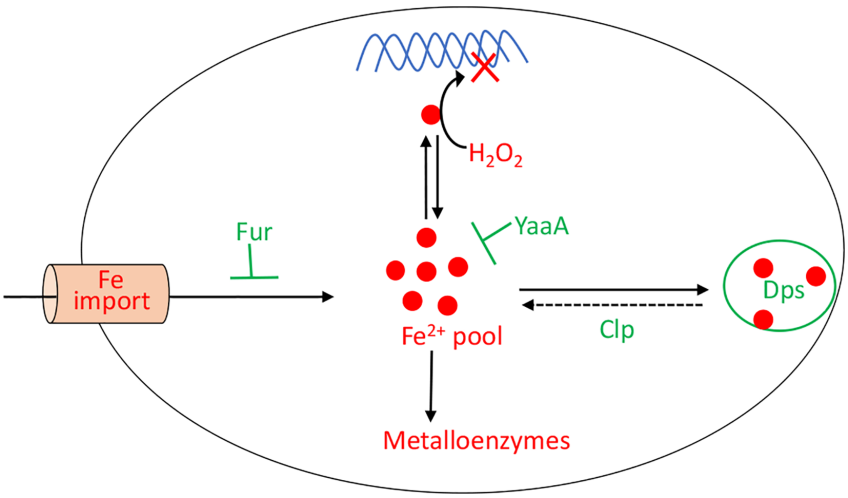


**FIGURE 7** | OxyR control of the intracellular iron pool. In order to minimize DNA damage, OxyR decreases the intracellular iron pool by inducing Dps, YaaA, and Fur. The Clp system maintains a small residual iron pool to enable synthesis of iron-dependent enzymes. The $H_2O_2$-responsive PerR regulon in *Bacillus subtilis* also controls Fur and MrgA, which is a Dps homolog.

the oxidation of Fe(II) by $H_2O_2$ can deactivate Fur, potentially leading to the disastrous import of more iron. The induction by OxyR of higher levels of Fur seems to partially correct this problem (91). Finally, YaaA is a protein whose biochemical action is not understood but which demonstrably shrinks the iron pool (92). Mutants that lack any of these proteins—Dps, Fur, or YaaA—exhibit high levels of intracellular iron and suffer rapid DNA damage during protracted $H_2O_2$ stress.

While the drop in iron pools helps protect DNA, it creates a problem for the synthesis or repair of iron-cofactored enzymes. Iron cofactors comprise three main types—iron-sulfur clusters, mononuclear Fe(II) groups, and heme—and the OxyR system turns on adaptations to sustain the function of enzymes that use each. *E. coli* typically uses Isc-based machinery to build iron-sulfur clusters (93, 94), but during $H_2O_2$ stress the secondary Suf system is induced (95, 96) (**Figure 4**). For unknown reasons, this system works well when iron levels are low (97), making it better than the house-keeping Isc system, which is poisoned by $H_2O_2$ (21). The induction of a manganese importer (MntH) (98) enables mononuclear enzymes to become metallated by Mn(II) rather than Fe(II) (**Figure 3**). Manganese is not as efficient a catalyst as iron, but it remains available even as Dps sequesters iron, and it is unreactive with $H_2O_2$ and thereby enables the mononuclear enzymes to remain functional (13). Finally, continued heme synthesis is facilitated by the induction of HemH, which encodes ferrochelatase (99). This enzyme inserts ferrous iron into porphyrins to complete heme synthesis, but it could potentially become a bottleneck when Fe(II) levels are diminished. Induction of the enzyme helps to circumvent that problem.

Thus, maintaining a balance in the intracellular iron pool during $H_2O_2$ stress is challenging. The cells need to keep the levels low enough to avoid DNA damage, but not so low that the synthesis of Fe-dependent enzymes is inhibited. As one final gambit, *E. coli* uses the Clp protease system to maintain this delicate balance (27). The Clp proteins help to release some iron from Dps, allowing for the repair of [4Fe-4S] clusters (**Figure 7**). Interestingly, genetic data raise the possibility that the Clp proteins do so not by degrading Dps but perhaps by unfolding it.

Not all the members of the OxyR regulon have been explained. Glutaredoxin 1 is induced. This dithiol compound is capable of reducing protein disulfide bonds, and it helps to deactivate OxyR once the $H_2O_2$ stress has dissipated (75). However, the reasons for the induction of glutathione reductase and thioredoxin 2, which also reduce disulfide bonds, are less clear. It would be surprising if low-micromolar $H_2O_2$ directly oxidized typical protein thiols—the rate constants for these reactions are too low (37, 77). An alternative is that these systems repair [4Fe-4S] enzymes and mononuclear Fe enzymes: Cluster reactivation requires a dithiol *in vitro* (21), and the reactivation of mononuclear enzymes can require the reduction of an active-site disulfide (13). An alternative—described below—is that these sulfur reducing systems are useful if OxyR moonlights as a sensor of other thiol-derivatizing stresses.

In *E. coli* both the reduced and oxidized forms of OxyR repress *oxyR* itself by binding over its promoter; this action ensures that the titers of OxyR are controlled and do not change during $H_2O_2$ stress (76). Transcriptomic data suggest that oxidized OxyR may repress several additional genes, including those that encode the periplasmic disulfide bond chaperone DsbG, the ferric iron reductase FhuF, the inner membrane protein of unknown function YbjC, and the NADPH nitroreductase NfsA (83, 99). The significance of this regulation remains unknown.

Most members of the regulon have functions that either prevent injuries or allow the cell to tolerate them. The regulon is very successful at this: Whereas one micromolar of $H_2O_2$ in the

environment fully blocks the growth of an OxyR-deficient strain, a wild-type strain is able to adapt and grow in 10 micromolar or more (60). Indeed, a final member of the OxyR regulon, cytochrome c peroxidase, is a periplasm-facing membrane-bound enzyme that allows *E. coli* to actually exploit environmental $H_2O_2$ as a respiratory oxidant when oxygen and nitrate are unavailable (100) (**Figure 2**). It is likely that the enzyme plays a role at oxic/anoxic interfaces near the intestinal epithelium, where $H_2O_2$ may be formed and may diffuse into anoxic zones.

## OxyR IS MODIFIED DEPENDING ON THE ORGANISM

The OxyR system has been most fully studied in *E. coli*, where it is presumably adapted for the enteric environment. Similarly, other bacteria seem to have adapted the OxyR regulon to suit their particular niches; they exhibit differences in terms of the regulation mechanism, the number of OxyR homologs, and the identity of the genes in the regulon. *Porphyromonas gingivalis*, for example, encounters two types of environments: the oral cavity where the oxygen tension is high and the hemin concentrations are low, and the periodontal pockets, which contain mixed microbial communities that lower the oxygen levels and are bathed with proteins such as hemoglobin that serve as a source of hemin. Perhaps unsurprisingly, the OxyR protein in *P. gingivalis* senses both signals, as evidenced by the further activation of OxyR-regulated genes in a hemin-limited environment under anaerobic conditions (101). Interestingly, measurements of the OxyR regulon genes indicate that they are constitutively expressed. This observation is consistent with the idea that the OxyR protein has mutated into a locked-on form, presumably as an adaptation to their environment, which contains $H_2O_2$-generating lactic acid bacteria.

With a few exceptions (83), OxyR in *E. coli* predominantly acts as an activator. However, in a variety of other organisms, it acts as a repressor and/or an activator, sometimes of the same gene: Reduced OxyR is a repressor and oxidized OxyR is an activator for the catalases *katG* in *Burkholderia pseudomallei, katB* in *Shewanella oneidensis, kat* in *Neisseria meningitidis* and *Neisseria gonorrhoeae, cat* in *Corynebacterium diphtheriae*, and *katA* in *Pseudomonas aeruginosa* (102–108). In these organisms, *oxyR* mutants exhibit a higher basal level of catalase expression compared to wild-type cells, as measured by gene expression and protein measurements. The presence of $H_2O_2$ further increases these levels in a wild-type cell but not in an *oxyR* mutant, indicating both repressor and activator function. It is unclear why there is value in having OxyR act as a repressor in some organisms and as an activator in others (**Table 2**). Its action as both a repressor and an activator may create a step-function-like turn-on switch, as a modest amount of $H_2O_2$ stress may be inadequate to fully convert the OxyR population to an activator form, and the residual reduced enzyme may block the action of a subpopulation of oxidized protein. In organisms that are exposed to low, continuous levels of $H_2O_2$ stress, AhpCF may suffice to protect the cell from $H_2O_2$, but when $H_2O_2$ levels become high, a full commitment to catalase synthesis may be called for. Conversely, in other organisms it may be more beneficial to preemptively synthesize basal levels of catalase to guard against a sudden deluge of oxidative stress. Under those conditions, the basal expression can help with the initial stress, and the activation of OxyR can further increase the scavenging enzyme titers.

Organisms such as *Vibrio cholerae, Vibrio vulnificus*, and *Deinococcus radiodurans* each deploy two OxyR proteins. In *V. vulnificus*, a facultative anaerobe that occasionally encounters aeration, the two proteins are calibrated to sense different levels of $H_2O_2$ (123–125). The more sensitive OxyR (VvOxyR2) is activated by endogenous $H_2O_2$ that is formed when the cell is aerated, whereas the less sensitive OxyR (VvOxyR1) is only activated by an influx of exogenous $H_2O_2$ from the environment. Accordingly, VvOxyR2 induces a peroxidase (VvPrx2) that has a higher activity at lower $H_2O_2$ levels compared to a second peroxidase (VvPrx1) that is induced by VvOxyR1. VvPrx1 becomes necessary because high levels of

**TABLE 2** | Mechanism of OxyR control in different bacteria.

| Bacteria | Oxygen tolerance | OxyR control |
|---|---|---|
| *Acinetobacter baumannii* | Aerobe | Activator and repressor (109) |
| *Caulobacter crescentus* | Aerobe | Activator (110) |
| *Burkholderia psuedomallei* | Aerobe | Activator and repressor (104) |
| *Corynebacterium diphtheriae*<br>*Corynebacterium glutamicum* | Aerobe | Repressor (107, 111, 112) |
| *Deinococcus radiodurans* | Aerobe | Activator and repressor (113, 114) |
| *Pseudomonas aeruginosa* | Aerobe | Activator and repressor (102, 108, 115) |
| *Neisseria gonorrhoeae*<br>*Neisseria meningitidis* | Aerobe | Repressor (106)<br>Activator and repressor (103) |
| *Streptomyces coelicolor* | Aerobe | Activator (116) |
| *E. coli* | Facultative anaerobe | Activator and repressor (83) |
| *Haemophilus influenzae* | Facultative anaerobe | Activator (117, 118) |
| *Klebsiella pneumoniae* | Facultative anaerobe | Activator (119) |
| *Rhodobacter sphaeroides* | Facultative anaerobe | Activator (120) |
| *Magnetospirillum gryphiswaldense* | Facultative anaerobe | Activator (121) |
| *Salmonella typhimurium* | Facultative anaerobe | Activator (71) |
| *Serratia marcescens* | Facultative anaerobe | Activator (122) |
| *Shewanella oneidensis* | Facultative anaerobe | Activator and repressor (105) |
| *Vibrio cholerae*<br>*Vibrio vulnificus* | Facultative anaerobe | Activator (123–125) |
| *Bacteroides fragilis*<br>*Bacteroides thetaiotaomicron* | Anaerobe | Activator (126–128) |
| *Porphyromonas gingivalis* | Anaerobe | Activator (101) |
| *Tannerella forsythia* | Anaerobe | Activator (129) |

$H_2O_2$ can irreversibly over-oxidize the catalytic cysteine residue of VvPrx2. It is unclear why there are two OxyR proteins in *D. radiodurans*. The two proteins seem to regulate different genes: OxyR1 activates *katE* and represses *mntH* and *dps*, and OxyR2 represses *katG* and the hemin transport genes (113, 114).

In some bacteria the OxyR regulon includes genes that are unrelated to iron control or $H_2O_2$ degradation. Superoxide dismutase is regulated by OxyR in *P. gingivalis* and *Pseudomonas aeruginosa*, seemingly implying that superoxide stress occurs concomitant with $H_2O_2$ stress (101, 130, 131). Perhaps the obligate anaerobe *P. gingivalis*, like *Bacteroides thetaiotaomicron* (132, 133), generates toxic doses of both superoxide and $H_2O_2$ whenever it enters oxic environments; thus, it may use $H_2O_2$ as a proxy to detect aerated habitats. In contrast, *P. aeruginosa* is an aerobe—but its special feature is that in competitive environments it synthesizes pyocyanin, a redox-active compound which can produce both ROS, perhaps to poison competitors (134). This behavior may necessitate the simultaneous synthesis of both superoxide and $H_2O_2$ defenses lest *P. aeruginosa* also poison itself. Meanwhile, DNA-binding assays and transcriptional analyses have shown that in *Magnetospirillum gryphiswaldense* OxyR induces the synthesis of genes involved in magnetosome production (121). Magnetotactic bacteria make internal magnetic particles as a way to orient themselves and dive into deeper water where there is less oxygen to impair these oxygen-sensitive bacteria; one infers that $H_2O_2$ is an environmental signal that triggers this defensive taxis. Together, these data indicate that OxyR has been adapted by different organisms in ways that fit their unique environmental niche.

A key question is whether OxyR plays a leading role in defending bacteria from the $H_2O_2$ that phagocytes produce as part of the cell-based immune response. Calculations predict phagosomal $H_2O_2$ levels (47, 66) that are adequate to activate OxyR, at least in *E. coli* (60), and local $H_2O_2$ levels could conceivably rise far higher in contained environments, such as abscesses. Infection data support this idea. OxyR is important in the colonizing ability of pathogens such as *E. coli* O1:K1:H7, *Bacteroides fragilis*, and *Hemophilus influenzae*: Mutants lacking *oxyR* were unable to colonize animal models or to induce abscesses in competition assays where they were mixed with wild-type cells (118, 126, 135). Biofilms can also shelter bacteria from external stressors—perhaps including $H_2O_2$ that is produced by an inflammatory response. The OxyR responses of *Serratia marcescens*, *Neiserria gonorrhoeae, Klebsiella pneumoniae*, and *Tannerella forsythia* mediate biofilm formation, a process that helps the bacteria to persist in hosts (119, 122, 129, 136). The OxyR system is required for swarming motility and the production of exotoxins by *P. aeruginosa*: *oxyR* mutants were non-motile on plates and were unable to inhibit dendritic cell proliferation (134, 137). However, while these observations demonstrate a role for OxyR in coping with host environments, they do not directly implicate the host response as the source of the $H_2O_2$ stress. Indeed, models of urinary tract infection demonstrated that *oxyR* mutants of *E. coli* were unsuccessful at colonization—but this phenotype persisted in a host that lacked its phagocytic NADPH oxidase (135). Presumably growth of the mutant was inhibited by $H_2O_2$ that was created by other environmental sources, such as competing lactic acid bacteria.

## THE THIOL-SENSING MECHANISM OF Yap1p

The model eukaryote *Saccharomyces cerevisiae* also activates a defensive response when it senses hazardous $H_2O_2$ in its environment—but although it depends upon a thiol-based sensor to do so, the sensor is unrelated to OxyR. Yap1p is the key transcription factor (138). Its amino-terminus has a bZip DNA-binding domain, but in the absence of $H_2O_2$, Yap1p shuttles between the nucleus and the cytoplasm (**Figure 8**). However, when $H_2O_2$ levels rise, Yap1p is indirectly activated. The cytoplasmic Gpx3 is a glutathione peroxidase whose catalytic Cys36 residue is alternatively oxidized to a sulfenic acid by $H_2O_2$ and reduced to the thiol by glutathione, with the average redox state dictated by the level of $H_2O_2$. This sulfenic acid form can react with the C598 residue of Yap1p, which is part of the cysteine-rich domain in the N-terminus, to form an interprotein disulfide intermediate. Subsequent thiol-disulfide exchange reactions lead to the formation of an intramolecular disulfide bond between Yap1 C303 and C598, causing global conformational change. As a result, the Yap1 nuclear export signal is hidden, which blocks its interaction with the nuclear exporter Crm1 (139–141). The resultant nuclear localization of Yap1p results in the activation of several genes. Thus, unlike OxyR, the cysteine residues of Yap1p do not directly react with $H_2O_2$. This difference may ensure that Yap1p stays activated even after migrating into the nucleus, where the $H_2O_2$ may not be as high as in the cytoplasm.

In broad outline, the membership of the Yap1p regulon overlaps with that of the OxyR regulon (**Table 3**). These proteins scavenge $H_2O_2$, change iron levels, and influence the thiol status of the cell (146, 147). Yap1p induces several peroxidases, including Ahp1, Gpx2, and Tsa1, that scavenge cytosolic $H_2O_2$; it is currently unclear why there are three such systems. The glutathione reductase *GLR1* is induced to reduce the glutathione disulfide that is formed when Gpx2 reduces $H_2O_2$. Additionally, Yap1p also drives synthesis of Ctt1, a cytosolic catalase. The mitochondrial iron exporter Mmt1 is also induced. It has been hypothesized that iron is exported in order to avoid $H_2O_2$ damage to the mitochondrial DNA (145). Alternatively, the flow of iron into the cytosol may help the repair of Fe-S clusters of $H_2O_2$-sensitive enzymes, such as LeuCD, that are localized there. Similar to OxyR, Yap1p is ultimately turned off by a thioredoxin system that consists of thioredoxins Trx1 and Trx2 and thioredoxin reductase Trr1 (148).

The fission yeast *Schizosaccharomyces pombe* features an interesting orthologue of the Yap1 system: Nuclear localization of the Pap1 transcription factor is accomplished when it receives

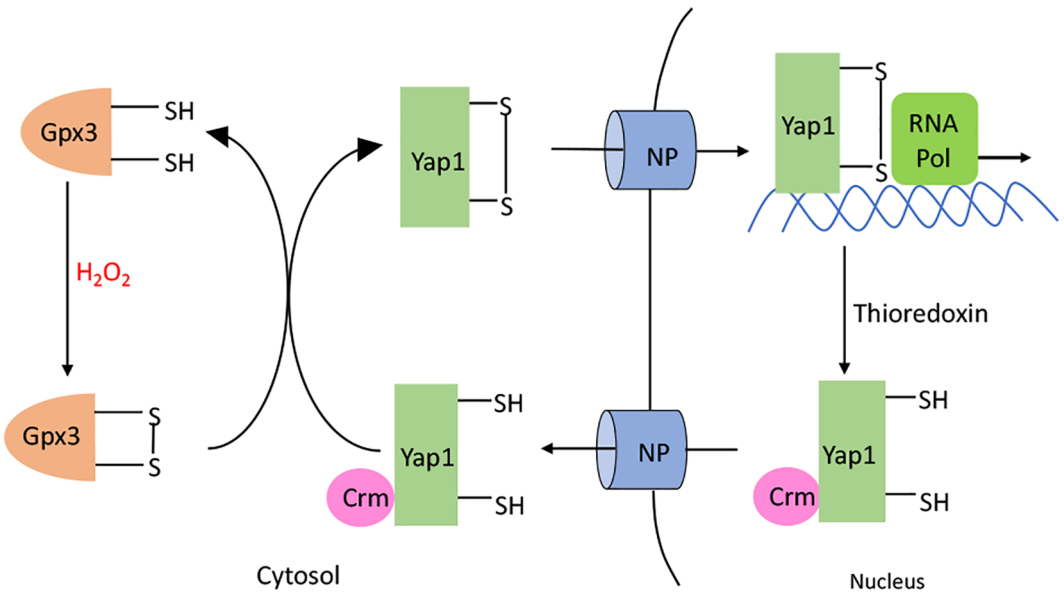


**FIGURE 8** | Yap1p activation. $H_2O_2$ oxidizes the C36 residue of glutathione peroxidase (Gpx3). The resulting sulfenate interacts with C598 in the C-terminal domain of Yap1p to form a intermolecular disulfide bond. Subsequent thiol-disulfide exchange reactions produce C303-C598 and C310-C629 disulfide bonds in Yap1p. Yap1p accumulates in the nucleus, leading to the activation of the Yap1p regulated genes. When $H_2O_2$ diminishes, Yap1p is reduced by the thioredoxin system; this change makes its nuclear export signal accessible to Crm, causing Yap1p to be transported back out of the nucleus.

**TABLE 3** | Genes induced by Yap1p during hydrogen peroxide stress in yeast.

| Gene | Function | Role during $H_2O_2$ stress |
|---|---|---|
| *AHP1* | Cytoplasmic alkyl hydroperoxidase | Scavenge $H_2O_2$ (142–144) |
| *GPX2* | Cytoplasmic glutathione peroxidase | |
| *TSA1* | Cytoplasmic thioredoxin peroxidase | |
| *CTT1* | Cytoplasmic catalase T | |
| *MMT1* | Mitochondrial iron exporter | Reduce the mitochondrial iron pool (145) |
| *GSH1* | Cytoplasmic glutamylcysteine synthetase | |
| *GLR1* | Cytoplasmic glutathione reductase | Maintain the thiol status (142, 146) |
| *TRX2* | Cytoplasmic thioredoxin | |
| *TRR1* | Cytoplasmic thioredoxin reductase | |

a disulfide bond from thiol peroxidase [reviewed in (149)]. One intriguing feature of its regulon is that it includes not only familiar $H_2O_2$ defenses but also drug-resistance genes. This feature raises the possibility that $H_2O_2$ stress is frequently imposed upon *S. pombe* by natural antibiotics, much as the linkage of drug pumps and SOD to the SoxRS system of *E. coli* reveals that redox-cycling drugs are a natural source of superoxide stress.

## THE Fe-BASED SENSING MECHANISM OF PerR

Some bacteria rely on a thiol-independent mechanism of $H_2O_2$ sensing. Unlike OxyR and Yap1p, the transcriptional repressor PerR takes advantage of the reaction between Fe(II) and $H_2O_2$ to detect the stress. Most extensively studied in *Bacillus subtilis* (150–152), PerR is a Fur homolog containing a structural zinc site and a regulatory metal binding site. It was probably easy to evolve Fur to sense $H_2O_2$ stress: Fur:Fe(II) already reacts with $H_2O_2$, and Fur and PerR are sufficiently similar in primary sequence that genomic inspection cannot reliably distinguish the two.

PerR acts as a dimeric repressor when it is bound to either Mn (II) or Fe(II). Under most growth conditions, PerR has a greater binding affinity for $Fe^{2+}$. However, in iron-limited medium that has been supplemented with manganese, PerR binds to Mn(II). The identity of the metal is functionally important, as Mn-bound PerR does not react with $H_2O_2$. This is probably by evolutionary design, as manganese-rich/iron-poor cells are intrinsically less vulnerable to $H_2O_2$. Because manganese supplants iron in mononuclear enzymes and a paucity of iron precludes much DNA damage, cells need not waste resources defending themselves against $H_2O_2$. In contrast, Fe-bound PerR reacts with $H_2O_2$ (**Figure 9**) with a rate constant of $10^5$ $M^{-1}$ $s^{-1}$, which approximates that of OxyR (153); the similarity in rate constants suggests that the same level of $H_2O_2$ may be toxic in *B. subtilis* as in *E. coli*. The reaction oxidizes two of the His ligands bound to iron, forming 2-oxo-histidine; because the oxidized ligands cannot bind metal and the oxidation cannot be reversed, the repressor is permanently inactivated (154). Intriguingly, *in vivo* studies have shown that the majority of PerR in *Staphylococcus aureus* ($PerR_{SA}$) is present in the oxidized form during aerobic growth, whereas this is not true of the *B. subtilis*

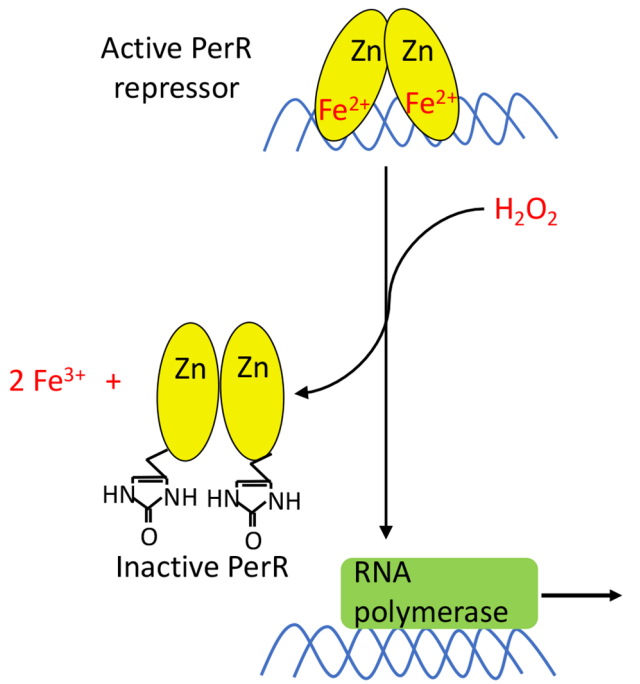


**FIGURE 9** | PerR activation. PerR is a dimeric DNA-binding protein, and it binds two metal ions per monomer. The first ion is a structural $Zn^{2+}$ that is necessary for dimerization and structural integrity. The second metal ion enables DNA binding, and can either be $Fe^{2+}$ or $Mn^{2+}$. Only PerR bound to $Fe^{2+}$ is responsive to $H_2O_2$. The oxidation of $Fe^{2+}$ by $H_2O_2$ generates a localized hydroxyl/ferryl radical, which irreversibly oxidizes either of two His ligands (H37 or H91) to form 2-oxo-histidine. Metal binding is blocked, PerR dissociates from promoter sites, and the regulon is induced.

PerR ($PerR_{BS}$) (155). When $PerR_{SA}$ and $PerR_{BS}$ were alternately expressed in the same organism, the KatA activity and transcript levels of PerR-regulated genes were higher with $PerR_{SA}$. One explanation is that $PerR_{SA}$ is more reactive than $PerR_{BS}$ and can sense lower concentrations of $H_2O_2$.

The inactivation of PerR results in the derepression of the PerR regulon, which again controls proteins that scavenge $H_2O_2$ and that lower the level of loose intracellular iron (156) (**Table 4**). AhpCF and KatA are induced to reduce the intracellular $H_2O_2$ levels. MrgA, which is a homolog of Dps, sequesters iron. Fur, as in *E. coli*, helps to reduce the intracellular iron levels by repressing iron import. The *hemAXCDBL* operon encodes the early steps of heme biosynthesis. Unlike *E. coli*, where OxyR induces the ferrochelatase HemH, *B. subtilis* PerR does not regulate the ferrochelatase.

Interestingly, the constitutive expression of the *B. subtilis* PerR regulon—in a *perR* mutant—causes trouble by excessively lowering the pool of intracellular iron (159). These mutants are more resistant to $H_2O_2$ but have trouble growing. It is unclear which enzyme-activity deficiency causes the poor growth. The iron deficiency was tracked to the combined repression of iron uptake by Fur, plus iron depletion due to the induction of KatA. Under these inducing conditions KatA becomes the single most abundant protein in the cell, comprising a whopping 10% of the total cell protein (159). This situation is reminiscent of OxyR-driven iron deficiency in *E. coli*; however, the latter is abated by induction of the Clp system (27). It seems, then, that whereas OxyR induction does not interfere with growth—and, indeed, can support it *via* the service of cytochrome c peroxidase—the induction of PerR is an emergency response that is incompatible with continued growth. Perhaps *B. subtilis* is wired to enter a period of stasis when exposed to $H_2O_2$ stress, with growth resuming only after the threat has passed, whereas OxyR allows *E. coli* to adjust and continue growing.

**TABLE 4** | Genes repressed by PerR in *B. subtilis* (157, 158).

| Gene | Function | Role during $H_2O_2$ stress |
|---|---|---|
| *ahpCF* | Alkyl hydroperoxidase | Scavenge $H_2O_2$ |
| *katA* | Catalase | |
| *mrgA* | Dps homolog | Reduce the intracellular iron pool |
| *fur* | Repressor of iron import | |
| *hemAXCDBL* | | Heme synthesis |

After $H_2O_2$ stress, the inactivated PerR is degraded by the protease LonA, and the repression of the PerR regulon is restored when the newly synthesized PerR binds either Mn or Fe (160).

## BACTERIA USE PerR DIFFERENTLY BASED ON THEIR NICHE

Similar to OxyR, PerR has also been adapted by bacteria to fit their particular niches. Differences have emerged in the types of PerR, what it senses, and the genes that it controls. Most bacteria have a single PerR regulator, such as *Staphylococcus aureus, Streptococcus pyogenes, Enterococcus faecalis*, and *Helicobacter hepaticus* (161–165). On the other hand, *Bacillus Licheniformis* has one PerR and two PerR-like proteins, both of which can sense $H_2O_2$ by histidine oxidation (166). Other bacteria contain both OxyR and PerR, including *N. gonorrhoeae, B. thetaiotaomicron*, and *D. radiodurans* (133, 167, 168); it is not yet clear why they would require both sensing systems.

The importance of PerR in different organisms may reflect the circumstances under which these bacteria experience $H_2O_2$ stress. Low-level aeration induces the PerR regulon of the anaerobe *Clostridium acetobutylicum*, perhaps due to endogenous $H_2O_2$ formation (169); as oxygen levels rise, induction of the regulon is critical for cell survival. In *Campylobacter jejuni*, a microaerophile that lacks SoxRS and OxyR homologs, the superoxide dismutase *sodB* is induced in *perR* mutants (170). These observations indicate that PerR may not be limited to defending cells against only $H_2O_2$.

Surprisingly, it has been shown that *perR* mutants of *S. aureus*, *S. pyogenes*, and Group A *Streptococcus* have lower virulence and lower intracellular survival in infected macrophages (161, 164, 171, 172), even though the derepression of the PerR regulon might be expected to induce defenses against the oxidative stress these bacteria encounter in their hosts. It is possible that the constitutive induction of the PerR regulon causes secondary growth defects similar to what is

seen in *B. subtilis* (159). If so, it may explain why the *perR* mutants of these pathogens are unable to colonize their hosts. In *S. aureus* and *Staphylococcus epidermidis*, PerR represses the expression of ferritin under low-iron conditions where PerR binds to Mn, and induces it in the presence of iron, indicating that like in *B. subtilis*, PerR can regulate metal homeostasis independently of oxidative stress (161, 173).

## DO OxyR, Yap1p, AND PerR USEFULLY DETECT OTHER STRESSORS?

The reactive sensors of OxyR and PerR—a hyperreactive thiol and Fe(II), respectively—can be modified by reactive species other than $H_2O_2$, and this observation raises the question of whether these transcription factors profitably respond to these other stresses. The effectors that have been examined most closely are nitric oxide (NO) and disulfide stress.

cesses (174, 175), and it is deliberately generated at toxic levels by macrophages as part of the cell-based immune response (176). It is a radical species that can pair with the unpaired d-orbital electrons of iron; as a result, NO binds heme, exposed iron-sulfur clusters, and mononuclear iron, potentially inhibiting the enzymes that possess these cofactors (177–180). Many bacteria use NO-sensing transcription factors to control the synthesis of NO scavenging enzymes. In *E. coli*, NorR is a Fe(II)-based regulator that induces the NorVW NO reductase, while NsrR is a [2Fe-2S]-containing transcription factor whose binding by NO triggers the induction of nitric oxide dioxygenase (Hmp) (181, 182). NsrR also appears to regulate a more expansive regulon, although the roles of other members are less clear (183). The *Vibrio fischeri* NsrR regulates an alternative oxidase that is more resistant to inhibition by NO than are conventional respiratory oxidases; thus, this feature of the NsrR regulon allows this squid symbiont to sustain its respiration despite the NO that is generated by its host (184). NO has a second route of toxicity, too: Its reaction with superoxide, which is also produced by macrophages, forms peroxynitrite ($ONOO^-$), a potent univalent oxidant that can penetrate into phagocytosed bacteria (185).

The Stamler group has presented evidence that OxyR also provides protection against NO stress (186, 187). Null mutants grew poorly during anaerobic respiration of nitrate, a process that might release some NO. Notably, the sensory cysteine of OxyR was nitrosylated, a modification that appeared to activate OxyR so that it induced a set of genes distinct from the conventional $H_2O_2$-driven response. The *hcp* operon was among those genes, and this group has proposed that Hcp contributes to the broader nitrosylation of cellular proteins, in a way that protects cells from nitrosative stress. The chemistry by which NO would chemically derivatize the OxyR thiol is not clear; NO is a radical species, so an oxidant, perhaps iron, needs to be involved to absorb the extra electron. Derivatization by Hcp is plausible; nitrosothiols readily react with activated cysteine residues, including that of OxyR, and this modification can perturb its behavior and has even been shown to initiate catalase synthesis—although it would seem to lack value in this situation.

However, other *in vivo* studies have elicited contradictory results. Chemostat cultures of *E. coli* that were grown anaerobically in the presence of 5 μM NO induced genes associated with NorR, NsrR, and Fur but not OxyR (188). Studies that have used even higher concentrations of NO sources, such as 1 mM acidified $NaNO_2$, activated NO-detoxifying systems such as *hmpA, norV*, and *norW*, but not OxyR (189). It is possible that the identity and dose of these nitrosative stressors as well as the growth conditions contributed to the discrepancies.

The role of Yap1p in protecting yeast from nitrosative stress is also unclear. Exogenous nitrosoglutathione elicited the synthesis of superoxide dismutase and catalase, and this response depended upon Yap1p (190). However, a study that used a nitric oxide donor did not detect this effect (191). The possibility exists, then, that the effects of NO and/or nitrosothiols upon OxyR and Yap1p are adventitious. Similarly, exogenous NO can react with the Fe(II) in bacterial PerR and, by inactivating the repressor, trigger induction of its regulon (192). However, members of this regulon do not provide any obvious route to remediate this stress, and so it seems likely that effect is merely incidental to the iron-binding activity of NO.

"Disulfide stress" is a term attached to conditions that create and disseminate disulfide bonds among cellular proteins. In many studies it is imposed by exposing microbes to diamide, a manmade reagent designed to create disulfide bonds from cellular thiols (193). In some bacteria diamide elicits defensive responses that include the induction of redoxin-based disulfide-reducing systems; these regulons (194, 195) are independent of the systems that detect and suppress $H_2O_2$ stress. Diamide can activate OxyR inside *E. coli*, but very high doses are needed (75). One wonders, then, what the natural circumstances are that trigger "disulfide stress" responses. Further, because OxyR-controlled redoxins have not yet been assigned a role in defraying $H_2O_2$ stress, it is formally possible that the sensory thiol of OxyR serves a second purpose of detecting and defusing thiol-targeting electrophiles.

Thus far, the only condition under which disulfide stress is known to naturally occur in *E. coli* is during periods of rapid cystine import (196). This situation arises when sulfur-limited cells, which induce all forms of sulfur importers, encounter cystine. Gross over-import of cystine results, and disulfide-exchange reactions cause disulfide bonds to be transferred from the imported cystine to cytoplasmic proteins. OxyR is modified, and it induces its regulon. Both the thioredoxin and glutaredoxin systems that it induces act to minimize the disulfide stress. Disulfide stress can also be imposed by exposing cells to antimicrobial plant compounds such as diallyl thiosulfinate and diallyl polysulfanes; the induction of *ahpC*, *trxA*, and *trxC* results (197). It seems unlikely that *E. coli* naturally encounters these chemicals.

Similar stresses can activate Yap1p of yeast. It is directly modified by diamide—without the mediation of Gpx3—and the $H_2O_2$-sensing C303 is not involved in the resultant conformational change (198). Instead, disulfide linkages are formed between C598 and C620, C620 and C629, and C598 and C629. Glutathione reductase and thioredoxin are subsequently induced. However, broadly speaking, the activation of these systems by disulfide-generating agents—and perhaps by nitrogen species—has

attracted a fair amount of attention without compelling evidence that these outcomes are not accidental.

## WHAT'S NEXT?

To date, the mechanisms by which $H_2O_2$ poisons bacteria have been explored primarily in test tubes. From those studies we have learned that $H_2O_2$ stress is iron-focused, driven by reactions with enzymic iron-sulfur clusters and Fe(II) prosthetic groups and with the pool of loose iron. The high rate constants of these reactions, and the abundance of vulnerable enzymes, means that low-micromolar concentrations of $H_2O_2$ suffice to bring bacterial growth to a halt. The known defenses remediate the same injuries that have been discovered, which provides confidence that the overall picture has come into shape. In *E. coli*, the most-examined microbe, it appears that the endogenous levels of $H_2O_2$ in the fully aerated bacterium falls just short of the threshold for $H_2O_2$ toxicity—and for the induction of emergency responses. Those responses, then, evidently exist to shield the cell from external $H_2O_2$.

The next step is to figure out how this information translates to real-world environments. The broad distribution of $H_2O_2$ defenses, and in particular of inducible defenses, suggests that $H_2O_2$ stress is a pervasive phenomenon that extends, at least episodically, to most biological habitats. Yet the actual circumstances and severity of oxidative stress remain poorly understood. Plausible sources of stress range from the redox collision that occurs at oxic/anoxic interfaces to the oxidative burst of mammalian and plant defenses. We infer that different microbes may encounter this stress in different circumstances, as the defensive regulons have been modified to suit their specific situations. The rationale for these differences is not always clear; in particular, we do not yet understand why some organisms use OxyR as a sensor, why others use PerR, and why a third set employ both. Thus, if we are to fully understand oxidative stress, we will need to continue to expand these studies beyond *E. coli* and yeast, and we will need to evaluate the intensity of $H_2O_2$ stress and the role of these systems in natural habitats.

For many microbiologists, the key questions concern whether host-generated $H_2O_2$ plays a role in suppressing invasion by most bacteria—and, if it does so, by which strategies dedicated pathogens manage to circumvent this stress. It may seem intuitively obvious that phagocytic $H_2O_2$ is a potent defense, but back-of-the-envelope calculations suggest that the $H_2O_2$ levels may not rise as high as workers have sometimes assumed. Part of the difficulty here is that phagocytic action has typically been studied using as prey the same professional pathogens that have, by definition, developed ways to elude the toxicity. We endorse the idea of studying the process using the 99% of bacteria that phagocytes efficiently kill.

## AUTHOR CONTRIBUTIONS

AS is the first author and JI is the last author. Both authors have contributed to the writing. All authors contributed to the article and approved the submitted version.

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REVIEW
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# Raising the 'Good' Oxidants for Immune Protection

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Redox medicine is a new therapeutic concept targeting reactive oxygen species (ROS) and secondary reaction products for health benefit. The concomitant function of ROS as intracellular second messengers and extracellular mediators governing physiological redox signaling, and as damaging radicals instigating or perpetuating various pathophysiological conditions will require selective strategies for therapeutic intervention. In addition, the reactivity and quantity of the oxidant species generated, its source and cellular location in a defined disease context need to be considered to achieve the desired outcome. In inflammatory diseases associated with oxidative damage and tissue injury, ROS source specific inhibitors may provide more benefit than generalized removal of ROS. Contemporary approaches in immunity will also include the preservation or even elevation of certain oxygen metabolites to restore or improve ROS driven physiological functions including more effective redox signaling and cell-microenvironment communication, and to induce mucosal barrier integrity, eubiosis and repair processes. Increasing oxidants by host-directed immunomodulation or by exogenous supplementation seems especially promising for improving host defense. Here, we summarize examples of beneficial ROS in immune homeostasis, infection, and acute inflammatory disease, and address emerging therapeutic strategies for ROS augmentation to induce and strengthen protective host immunity.

**Keywords: reactive oxygen species, NADPH oxidase, microbiota, host defense, immune signaling, redox medicine, lactobacilli, glucose oxidase**

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## INTRODUCTION

Reactive oxygen species (ROS) is a generic term referring to oxygen-derived compounds capable of reacting with biological molecules through an oxidation-reduction ("redox") mechanism. ROS include a set of highly reactive radicals (e.g., superoxide anion, hydroxyl radical) as well as non-radical species [e.g., hydrogen peroxide ($H_2O_2$)] produced enzymatically or chemically in eukaryotic and prokaryotic cells. In mammals, one of the main intracellular sources of ROS is the mitochondrial electron transport chain (ETC) during the establishment of the proton-motive force. Premature electron leakage to molecular oxygen occurs mainly from complex I and III of the ETC during the serial transfer of electrons, causing superoxide formation that is converted to $H_2O_2$ by superoxide dismutase (SOD) (1, 2). In contrast to ROS generation as by-product of aerobic metabolism, of oxidation of fatty acids or proteins, or of enzyme-substrate reactions (e.g., xanthine oxidase, lipoxygenase, cyclooxygenase, monoamine oxidase), the NADPH oxidase family (NOX/DUOX) is solely

dedicated to ROS production and catalyzes the reduction of molecular oxygen to superoxide or $H_2O_2$. Seven oxidase isoforms have been characterized in humans, differing in their catalytic core (NOX1-5 and DUOX1-2), their requirement for additional components for complex stabilization and/or enzyme activation, their subcellular localization and in tissue specificity (3, 4). The NADPH oxidase prototype is the NOX2 enzyme, an essential superoxide source for pathogen defense by neutrophils and macrophages. The rapid increase of superoxide ('respiratory burst') is accompanied by the formation of dismutation and adduct products, including hypochlorite (generated by myeloperoxidase, $H_2O_2$ and chloride ion) and peroxynitrite (spontaneous reaction of superoxide with nitric oxide), both strongly oxidizing and nitrating compounds that drive pathogen killing in concert with activated proteases (5). Tight regulation of ROS generation is essential, as ample and uncontrolled production of highly reactive species over an extended time period will cause irreversible redox modifications on biomolecules, thus promoting oxidative damage.

ROS is not only produced during host defense or in proinflammatory situations. Superoxide and $H_2O_2$ are continuously generated, converted, and degraded in physiological conditions (6, 7). $H_2O_2$ as a key intracellular messenger mediating signal transduction in all cell types maintains homeostasis and physiological host responses (8, 9). $H_2O_2$-initiated redox signaling is essential for basic cellular processes (e.g., proliferation, migration, secretion), thereby supporting complex functions in mucosal immunity (e.g., barrier integrity, host-microbiota communication, host defense, chemokine/cytokine generation, wound repair), driving innate immune functions and regulating adaptive immunity. By aquaporin-facilitated diffusion across the plasma membrane or by release from intracellular organelles (e.g., redoxosomes, microvesicles, mitochondria), $H_2O_2$ mediates directly or more commonly *via* redox relays the reversible oxidation of distinct amino acids, modifying the structure and function of targeted proteins and consequently the activation state of the associated signaling pathways. In mammals, thiol oxidation of certain reactive cysteine residues occurs within redox-sensitive proteins, including phosphatases and tyrosine kinases, leading to activation or inactivation of the targeted protein (2, 10). Redox signaling also drives the antioxidant response to prevent excessive ROS production. A notable conduit for transcriptional activation of antioxidant genes is the KEAP1-NRF2 pathway. $H_2O_2$-mediated oxidation of the redox-sensitive adaptor KEAP1 disrupts its cytoplasmic complex with NRF2, promoting nuclear translocation of newly synthesized NRF2 which will bind to antioxidant response elements (ARE) located in the promoter region of NRF2 target genes such as NAD(P)H quinone oxidoreductase 1 (NQO1), heme oxygenase 1 (HMOX1), glutamate-cysteine ligase (GCL) and glutathione S transferases (GSTs) (11, 12).

Coupled oxidant-antioxidant pathways have been conserved throughout evolution. For example, in bacteria dedicated transcriptional regulators (e.g., OxyR, OhrR, PerR, SoxR) sense ROS/RNS in order to induce an appropriate detoxification response (e.g., radical scavenging and metal sequestrating systems), and to maintain intracellular oxidant levels within a safe limit (13, 14). The $H_2O_2$ sensing transcriptional regulator OxyR is a well-studied example in mainly Gram-negative bacteria. When intracellular $H_2O_2$ exceeds a certain threshold in *Escherichia coli* [~ 0.1-0.2 μM (15, 16)], oxidation of OxyR on two cysteine residues prompts a conformational change due to intramolecular disulfide bond formation, activating the expression of OxyR-dependent antioxidant defense genes (17, 18). At mucosal surfaces physiological $H_2O_2$ levels mediate the intricate redox communication between commensal microorganisms and the host barrier tissue. For instance, bacteria can induce or hinder physiological host signaling, either by releasing $H_2O_2$ in the vicinity of host epithelia (e.g., lactobacilli) or by altering host enzyme-mediated ROS generation *via* secreted compounds or physical interaction with epithelial barrier cells (19–21). Likewise, $H_2O_2$ released by host cells can alter bacterial signaling in response to a pathogenic insult, particularly in low oxygen environments (22, 23).

It is important to distinguish these beneficial redox-mediated mechanisms supporting health from conditions promoting supraphysiological level of ROS and the resulting oxidative damage. To ensure physiological conditions, the activation of NADPH oxidases is usually regulated by multiple inputs (e.g., transcriptional, posttranslational, metal ions, nucleotides) and ROS generation is coupled with decomposing and scavenging systems (e.g., catalase, peroxidases, antioxidants) (2, 4, 21, 24). Ongoing perturbation of ROS levels, either at high or low range, has been associated with various pathophysiological states. Persistent increase of highly reactive species (e.g., superoxide anion, hydroxyl radical, peroxynitrite) has been associated with chronic inflammatory and hyperglycemic (e.g., rheumatoid arthritis, inflammatory bowel diseases, type 2 diabetes), tumorigenic and neurological (e.g., Parkinson's, Alzheimer's) diseases. Therapeutically decreasing ROS levels by non-specific enzyme inhibitors or antioxidants has been pursued for many decades (25, 26). However, the ill-defined and generalized action of these molecules limits their efficacy and, in some cases, exacerbated the underlying condition (27–32). More targeted approaches, namely inhibition of a specific ROS source, may provide more benefit. Conversely, permanently decreased ROS generation due to loss-of-function variants in genes encoding for NADPH oxidases or other ROS sources, or in genes providing an essential upstream trigger for a superoxide/$H_2O_2$ generating enzyme, have been linked to various pathologies, including recurrent microbial infections, chronic inflammatory diseases, and autoimmunity (33). In this case, a strategy to restore or enhance ROS may prove beneficial.

Understanding redox regulation of vital physiological processes and how alterations initiate or perpetuate disease is important for developing new therapeutic avenues. In addition to traditional therapeutic strategies aimed at counteracting excessive ROS, contemporary approaches should include preservation or even the elevation of certain oxygen metabolites for physiological purposes. This review will provide selected examples of beneficial ROS in protective host

immunity and will discuss emerging therapeutic strategies to preserve or augment ROS with the aim of re-establishing homeostasis and promoting host protection.

# IMPORTANCE OF OXIDANTS IN PROTECTIVE IMMUNITY

## Homeostatic Redox Signaling

Physiological ROS levels modulate many immune functions by redox-sensitive signaling. Not only $H_2O_2$, but also related second messengers such as nitric oxide and possibly peroxynitrite control signaling responses and thus these short-lived molecules represent an integral part of immune regulation (34–37). All redox-regulated pathways show analogous features, namely inhibitory or activating modifications on proteins and lipids (10, 38, 39), and are conserved across different cell types and organisms. Redox modifications can constrain or stimulate signaling pathways. An example for inhibitory oxidative modifications is dual specificity phosphatase 1 (DUSP1) which will be degraded after oxidation or S-glutathionylation of an active site cysteine, resulting in prolonged MAPK-induced proinflammatory gene transcription (40). Reversible oxidative inactivation of protein tyrosine phosphatase 1B (PTP1B) amplified interleukin (IL)-4 receptor activation (41). On the other hand, redox-dependent oxidation of two cysteine residues in the tyrosine kinase SRC caused structural changes that impacted regulatory tyrosines, enabling SRC kinase activation (42). In airway epithelial cells, epidermal growth factor receptor signaling was linked to activation of the NADPH oxidase DUOX1 and subsequent SRC oxidation (43). Kinase activation can also occur by oxidation-driven dissociation of an inhibitor-protein kinase complex as demonstrated for the complex between thioredoxin and apoptosis signal-regulating kinase 1 (ASK1) (44). The liberated form of ASK1 will then form multimeric complexes with other signaling mediators and positively regulate JNK/p38 MAPK pathways, while itself being further controlled by dephosphorylation and deubiquitination (45). More examples and details on redox regulation of signaling pathways can be found in recent reviews (1, 46).

The initiation of redox signaling occurs mainly after ligand stimulation of receptors (e.g., Toll-like receptors (TLR), G protein-coupled receptors (GPCR), NOD-like receptors (NLR), Fc receptors, chemokine and cytokine receptors, and others) and may include one or several ROS sources concomitantly or consecutively, depending on the context. For instance, redox regulation triggered by TGF-β involves the NADPH oxidase NOX4 as well as mitochondrial ROS (mROS) (47). NADPH oxidases seem to be closely associated with TLR-stimulated pathways where oxidase deficiency either inhibits or enhances host cell responses. Loss of NOX1 in smooth muscle cells increased TLR2-mediated MIP-2 generation but impeded matrix metalloprotease 2 secretion and directed cell migration (48). In epithelial cells NOX4 was reported to associate with TLR4, regulating the transcription factor NF-κB (49). Loss-of-function mutations in any of the five subunits comprising the NOX2 complex cause the inherited immunodeficiency chronic granulomatous disease (CGD) (33, 50). One of the effects of CGD in the immune system is the altered responsiveness to TLR ligands, resulting in augmented cytokine generation and hyperinflammation. Neutrophils, monocytes, and dendritic cells isolated from CGD patients or Nox2 deficient mice generated increased levels of cytokines upon TLR2 or TLR4 stimulation (51–54). A hyperinflammatory phenotype with excessive secretion of pro- and anti-inflammatory cytokines was also observed in CGD B lymphocytes stimulated with TLR7 and TLR9 agonists (55). This cytokine overproduction was associated with hyperactivation of p38 MAPK signaling, which may indicate loss of inhibitory feedback signaling, likely involving phosphatases, when the NOX2 enzyme is inactivated. Increased levels of IFNγ and IL-18 in CGD patient tissues was in part traced to macrophages remaining in a proinflammatory M1 state (56). Innate immune cells derived from CGD patients or mice also showed an exuberant IL-1 response to various inflammasome activating stimuli, while the NRF2-controled antioxidant response was dampened (57–59). The hyperinflammatory CGD phenotype in mice and man is intensified by increased neutrophil recruitment to sites of infection and tissue damage, which was recently linked to feed-forward amplification of neutrophil generated leukotriene B4 upon pulmonary zymosan challenge (60). Hence, deficiency in NOX2 enzyme activity creates a proinflammatory environment that is associated with several chronic inflammatory conditions and can predispose to autoimmune disease (61). The molecular mechanisms underlying various facets of CGD hyperinflammation are still not resolved, but NOX2-derived superoxide seems to be required for the precise regulation of many immune cell functions including gene transcription, autophagy, efferocytosis, and dendritic cell-mediated antigen presentation (61–64).

In recent years, the role of mROS signaling in regulating both innate and adaptive immunity received increased attention (65, 66), although in many cases molecular mechanisms are not well characterized and mROS participation in signaling is mainly inferred by using mitochondria-targeted ROS probes and/or antioxidants. Chandel and coworkers linked early on a TNF receptor-TRAF pathway to an increase in mROS (67), and by now many different stimuli are recognized as triggers of physiological mROS generation (6). Here we summarize recent insights into mROS-mediated immune cell responses. Signaling cascades driven by TLRs stimulate not only NADPH oxidase activity, but also *via* TRAF6 the mitochondrial matrix protein ECSIT that stimulates superoxide production from ETC complex I (68). Others reported recently that pro-inflammatory cytokine secretion by *Listeria monocytogenes*-infected macrophages is contingent on mROS-induced disulfide bond formation in the IKK complex regulator NEMO (69). Activation of the NLRP3 inflammasome by pathogen-associated or danger-associated molecular patterns (PAMPS/DAMPS) is controlled by mROS, resulting in release of the proinflammatory cytokines IL-1β and IL-18 (70, 71). The precise sequence of events that may include a

transcriptional priming step, oxidized mitochondrial DNA, and mROS-dependent interaction of NLRP3 with thioredoxin-interacting protein is not yet fully established (72). In macrophages NLRP3 inflammasome activation required xanthine oxidase generated superoxide upstream of mROS generation (73), while studies in superoxide deficient peripheral blood mononuclear cells derived from CGD patients revealed that NOX enzymes are not required for NLRP3-mediated IL-1β secretion (74, 75). Cytosolic retinoic acid-inducible gene I (RIG-I)-like receptors such as RIG-I and MDA5 sense and bind distinct features of viral RNA, then oligomerize, and associate with the adaptor protein MAVS at outer mitochondrial membranes and mitochondria-associated membranes. Oligomerization of MAVS recruits several effector proteins to form the MAVS signalosome, driving IRF3/IRF7-mediated transcription of type I/III interferon and NF-κB-induced proinflammatory cytokine expression. MAVS expression has been linked to NOX2 generated superoxide (76), while others reported functional interactions involving MAVS aggregation, mROS generation, and the cytochrome c oxidase component COX5B that couple the antiviral response to autophagy (77). The NLR family member NLRX1 cannot only interact with MAVS at the outer mitochondrial membrane but is also localized within the matrix and inner membrane of mitochondria where interaction with the ETC complex III associated protein UQCRC2 promotes mROS generation, thereby stimulating transcription factors and JNK MAP kinase (78). The signaling function of the immune response-induced mitochondrial interactome is clearly connected to mROS generation, in physiological and pathophysiological conditions, and many more aspects of mitochondrial immune signaling and connections between different ROS sources influencing each other in these processes can be expected in the future.

## Mucosal Barriers and Oxidants

The mucosal immune system in lung, gastrointestinal tract (GIT), urogenital tract, oral and nasal passages employs structural, chemical and immunological barriers to control the interaction with the adjacent microorganism- and noxious substance-rich environment. The importance of mucosal barriers as host defense mechanism is reflected in these multiple layers of protection together with the constant renewal of barrier epithelia, specialized protective measures such as secretion of mucins or surfactant, and highly efficient repair mechanisms. Superoxide and $H_2O_2$, generated by host enzymes or commensal bacteria, are crucial for various aspects of mucosal barrier maintenance as outlined here in selected examples.

The structural integrity of mucosal epithelial barriers relies on intercellular junctions, in particular tight junctions, and after a breach occurred, on rapid proliferation and migration of epithelial cells for wound repair. ROS regulate cytoskeletal dynamics such as oxidative modification of β-actin and tubulin, RhoA GTPase activation and deactivation depending on the context, redox-induced integrin and focal adhesion kinase (FAK) activation and oxidative modifications on other proteins involved in cytoskeletal rearrangements (79, 80). As many of these physiological processes have not yet been conclusively linked to a particular ROS source or studied in epithelial barriers, we will present here only results that identified the enzyme involved, were obtained by multiple approaches and if possible *in vivo*. In the GIT, superoxide produced by NOX1 controls colon epithelial cell proliferation and migration, in part by enhancing FAK phosphorylation due to oxidative inactivation of the tyrosine phosphatases LMW-PTP and SHP-2 that will initiate focal adhesion turnover, accelerate cell migration, and improve wound closure (81–85). Others reported NOX1-mediated oxidation of nucleoredoxin that released suppression of the canonical WNT-β-catenin pathway, resulting in transcription of growth promoting genes (86, 87). In airway epithelial cells DUOX was localized at the leading edge of migrating cells, augmenting wound healing (88). DUOX1 generated $H_2O_2$ regulated cell migration and epithelial repair in cells and in a naphthalene airway injury model by promoting epidermal growth factor receptor (EGFR)-STAT3 signaling (89, 90).

An important part of the physical gut barrier is the protective mucus layer, either single-layered in the small intestine and pulmonary tract, or 2-layered with a dense, impenetrable layer followed by a loose layer in the colon. Mucus is secreted and continuously renewed by goblet cells and is composed of mainly MUC2 mucin in the intestine, MUC1, MUC5AC and MUC6 mucins in the stomach, and MUC5AC and MUC5B mucins in the lung epithelium (91). NADPH oxidase generated $H_2O_2$ has been linked to intestinal mucin granule release by pathways involving endocytosis, autophagy, and NLRP6 signaling (92, 93). A severe colonic phenotype with partial loss of the dense mucus layer was only observed in mice with combined inactivation of Nox1-3 but not in single oxidase knockout mice (94), likely due to *in vivo* compensation by another oxidase (or ROS source) or by $H_2O_2$ producing lactobacilli colonizing the mucus layer. In the airways EGFR and IL-13 signaling promoted mucin gene induction (MUC5AC, CLCA1) *via* DUOX1 (95, 96), while others reported a TLR5-DUOX2 pathway regulating MUC5AC expression in nasal epithelium (97).

The structural reinforcement of the intestinal barrier is accompanied by chemical and immunological secretions. Immunological secretions are immunoglobulin A (sIgA) and various other antimicrobial factors including lysozyme, regenerating islet-derived proteins 3, and cationic peptides such as cathelicidins or defensins. Cationic peptides range from α-helical (e.g., LL-37) to β-sheet (e.g., β-defensins) conformation or random-coil structures and exert their activity by bacterial membrane disruption and immunomodulatory effects on host cells (98, 99). While their synthesis or secretion has not been linked to a ROS source, the proper folding of some bioactive cysteine-rich peptides such as β-defensin 3 require the oxidative formation of several disulfide bridges (100). Lipocalin-2 (LCN-2), secreted by intestinal epithelial cells or released from neutrophil secondary granules, is often used as a biomarker of inflammatory processes, but also limits bacterial growth by binding iron-loaded siderophores (101, 102). A recent report connected LCN-2 expression *via* atypical IκBζ-dependent gene

transcription to NOX1 activity in colonic epithelial cells and mice (103).

Mucosal barriers generate and release chemical compounds such as superoxide, $H_2O_2$, nitric oxide and peroxynitrite when pathogens or danger-associated molecules trigger host cell responses. These chemicals may also be released constitutively at low concentrations as repellent against commensal communities, promoting a mutualistic host-microbiota relationship essential for immunity (21, 104). This is supported by reports that enteric bacteria regulate NADPH oxidase activity by relatively undefined pathways (85, 105, 106), and vice versa that ROS generated by mitochondria or epithelial cells control the microbial population, thereby limiting the access of the microbiota to the immune compartment (19, 107). This scenario likely takes place in the small intestine with its loose, single mucus layer and may involve the oxidase DUOX2. DUOX2 expression is upregulated by microbiota, and its localization at the apical surface of villi is ideally suited for $H_2O_2$ release, thereby reinforcing the separation of host and microbial communities (19). Additionally, peroxynitrite generated by the combined activation of NOX1 and NOS2 may participate in the control of ileal homeostasis (108, 109). Similarly, $H_2O_2$ release by DUOX1/DUOX2 and subsequent lactoperoxidase-mediated conversion to secondary oxidants (i.e., HOSCN) may repel bacteria in the airways (110). Influenza A virus (IAV) infection stimulated $H_2O_2$ release from air-liquid interface cultured primary human airway epithelial cells in a calcium/flavoenzyme dependent manner, suggesting DUOX activation (111).

Chemical messengers are ideally suited to relay signals from the host to the microbiota and vice versa. This interkingdom communication will shape microbiota diversity and composition and provides a stimulus for homeostatic barrier host responses. While aberrant ROS production in inflammatory disease will increase the abundance of certain bacterial communities and may lower overall diversity, decreased levels or loss of NADPH oxidase-generated ROS, as observed in certain patient populations, will also favor dysbiosis, indicating that a tightly balanced mucosa-associated microenvironment is indispensable for gut homeostasis. While CGD is mainly associated with recurrent life-threatening infections, 40-50% of these patients will develop inflammatory bowel disease with some features of Crohn's disease. When comparing the microbiota of CGD patients to healthy individuals an increased abundance of mucus foraging *Ruminococcus gnavus* was noted (112). This study included only a limited number of patients (10 individuals) and might be skewed due to the required antibiotic maintenance of these patients. In a CGD mouse model (i.e., $p47^{phox}$ deficiency) the microbiome signature was altered by high abundance of *Akkermansia muciniphila* (113). This mucolytic bacterium is considered a beneficial probiotic, but can also disturb mucus homeostasis in the host, thereby acting as a pathobiont or aggravating inflammation induced by intestinal pathogens (114–116). In the ileum of Nox1 knockout mice increased abundance of Bifidobacteria and Turicibacter was detected (117). Global inactivation of the oxidases Nox1-3 in mice ($Cyba^{nmf333}$) resulted in increased abundance of Proteobacteria, Ruminococcaceae and *Mucispirillum schaedleri* (94). An increase of *M. schaedleri* was also noted in mice with combined Nod2/Nox2 deficiency (118). This mucus dwelling bacterium interfered with *Salmonella enterica* serovar Typhimurium virulence factor expression and decreased pathogen invasion in wildtype mice (119), but it also promoted inflammation in Nod2/Nox2 double knockout mice. Inactivation of all four murine Nox enzymes by global or intestinal epithelium restricted deletion of the essential Nox partner protein $p22^{phox}$ ($Cyba^{-/-}$, $Cyba^{Vil\text{-}cre}$) induced compensatory upregulation of $H_2O_2$ producing lactobacilli as a host protective, mutualistically beneficial mechanism (120). These results in mice suggest that $H_2O_2$ generation, mucus changes and overgrowth of mucus-dwelling or mucolytic bacteria are closely linked. Thus, ROS maintain and protect the stable microenvironment required for balanced and interconnected host-commensal cooperation and homeostatic barrier function.

## Specialized Roles of Oxidants in Innate Immune Cells

### Macrophage Polarization

The versatile role of ROS in regulating the intracellular signalosome in a constantly evolving microenvironment underpins their role in promoting polarization of immune cell populations and supporting specialized functions. A pertinent example is altering the polarization and activation state of macrophages in the broad categories of M1 (classical) and M2 (alternative), categorized by a proinflammatory, host defense connected phenotype versus an anti-inflammatory, tissue remodeling phenotype (121). NOX2 is expressed in M1 and M2 macrophages and regulates the transition between phenotypes in a context dependent manner. Nox2 deficient bone marrow derived macrophages (BMDM) upregulated STAT3 activation and anti-inflammatory cytokine expression (i.e., IL-10), while others reported no differences between wildtype and Nox2 knockout M2 macrophages (122, 123). Conflicting results in the literature stem likely from the range of protocols used for macrophage differentiation (e.g., conditioned media versus recombinant GM-CSF or M-CSF for various time periods, LPS content of fetal bovine serum) and the inclusion or omission of further Th1 or Th2 type polarization. M1 polarized BMDM express not only Nox2 but also Nox1 (124). When M-CSF differentiated BMDM or peritoneal macrophages derived from wildtype, Nox1, Nox2 or Nox1/Nox2 knockout mice were further polarized by LPS+IFNγ or IL-4+IL-10, only Nox1/Nox2 deficient macrophages exhibited reduced M2-type polarization while M1 polarization and pro-inflammatory functions were preserved (123). Expression of Nox4 in macrophages and its downstream effects seem to be determined by the microenvironment. Using a similar M1/M2 polarization protocol as described above Helfinger and coworkers observed in Nox4 deficient mouse macrophages amplification of the M1 phenotype, suggesting an anti-inflammatory role for Nox4 (125). On the other hand, NOX4 expression was induced in human monocyte-derived

macrophages by low-density lipoprotein (OxLDL) that stimulates a pro-inflammatory response (126), and in profibrotic M2 alveolar macrophages (127). A mechanistically poorly defined link between NOX4 and mROS has been proposed (i.e., ROS-induced ROS) that could play a role in conferring the divergent metabolic signatures observed in M1 versus M2 macrophage polarization (127–131). $H_2O_2$-mediated intercellular communication between various innate immune cells can also drive macrophage skewing. In vivo phenotypic conversion of pro-inflammatory to pro-resolving macrophages was dependent on neutrophil Nox2 and $H_2O_2$ generation, further confirming that activated neutrophils contribute to resolution of inflammation (132–135). While less characterized, neutrophils can, analogous to macrophages, polarize towards distinct phenotypes. N1 and N2 neutrophil populations are mainly defined by their functional phenotype with N1 considered pro-inflammatory and anti-tumorigenic, and N2 pro-tumorigenic. A role for ROS in this phenotypic conversion has not yet been defined.

### Dendritic Cell Function

Dendritic (DC) cells are antigen-presenting cells that are essential for inducing naïve T cell activation and effector differentiation. After taking up antigens and microbes by phagocytosis or endocytosis DCs generate MHC peptide complexes. For activation of CD4 + T helper cells, antigens are degraded in lysosomes for MHC class II presentation, while activation of CD8 + cytotoxic T cells requires that antigens are presented in MHC class I by cross-presentation that occurs *via* lysosomal and cytosolic pathways. Interaction of mature DCs with T cells takes place in secondary lymphoid organs such as lymph nodes, spleen, and Peyer's patches, initiating adaptive immune responses (136). Superoxide production by NOX2 is an intricate part of antigen processing, generation of MHC-peptide complexes and cross-presentation. Work by Amigorena and coworkers revealed that active NOX2 regulates the phagosomal pH in DCs by sustained superoxide generation, maintaining it close to the optimal pH 7.4 despite of proton import by V-ATPase. In DCs derived from CGD patients or CGD mice the phagosomal pH acidified and the cross-presentation of antigens was impaired (137, 138). In addition, NOX2 activity preserved antigens from proteolysis by inhibiting endosomal and lysosomal proteases via oxidation (139). Recent work connected NOX2 to antigen release from the endocytic lumen into the cytosol during cross-presentation. NOX2-derived superoxide caused lipid peroxidation, thereby disrupting membranes to enable antigen leakage from endosomes, a process that was impaired in DCs derived from CGD patients (140). Further study of a specialized subset of DCs, plasmacytoid DCs (pDCs), distinguished from conventional DCs by morphology and function, revealed that in this cell type antigen protection and cross-presentation is Nox1- and Nox2-independent. Here, mROS generated by pDCs after TLR7 stimulation promoted antigen cross-presentation and the capacity to trigger CD8+ T cells responses, as well as facilitating additional functions such as IFN-α production (141). Antigen presentation is also linked to macroautophagy that serves not only as mechanism for nutrient recycling and host defense against intracellular bacteria, but also for presenting MHC II molecules to CD4+ T cells. Recruitment of autophagy proteins to phagosomal membranes occurs when antigens are recognized by immune receptors such as TLRs, FcRs or dectin-1, resulting in LC3-associated phagocytosis (LAP) (142, 143). LAP requires NOX2-generated superoxide for recruitment and lipidation of the autophagy protein Atg8/LC3 to phagosomes, and fungal antigen storage in innate immune cells was compromised in CGD patients (144, 145). NOX2 activity was also involved in antimicrobial LAP in endothelial cells (146). Autophagy processes involved in cellular maintenance seem to be coupled to oxidase-derived ROS in many cell types including to NOX4 in cardiomyocytes and to a NOX family member in goblet cells (92, 94, 147), but putative ROS source(s) facilitating pathogen uptake due to LAP or other processes in epithelial cells, an important consideration for mucosal barrier host defense, have not yet been defined.

## Host Defense and Oxidants

Respiratory and intestinal barrier epithelia are the first line of defense against airborne or foodborne pathogens. When the initial protective mechanisms (e.g., colonization resistance, antimicrobial secretions, mucus layer) have failed, epithelial sensing pathways will be activated. In the mucosal compartment, sensing of conserved microbial motifs by epithelial cells induces ROS production due to upregulation and/or activation of mucosal NADPH oxidases (e.g., DUOX1/2 in airways, NOX1/DUOX2 in GIT) that participate in the antimicrobial response (97, 110, 148–150). Transient or stable expression of the NOX1 or DUOX2 complex in several epithelial cell lines decreased pathogen attachment and invasion without affecting bacterial viability (151, 152). NOX1 or DUOX2 mediated $H_2O_2$ release interfered with the pathogenicity of bacteria by inducing irreversible oxidative modifications in bacterial enzymes and proteins essential for maintaining virulence factor synthesis (22, 23). Duox inactivation in mice, achieved by deleting the essential partner protein Duoxa, increased gastric colonization with *Helicobacter felis*, reflecting the protective effect of $H_2O_2$ in host defense (153). Infection of these mice with *Salmonella* Typhimurium augmented systemic dissemination of the pathogen, suggesting that Duox is an integral component of the protective mucosal barrier in the small intestine (19). In murine airways Duox silencing increased the Influenza A virus (IAV) load by interfering with viral replication, at least in part by affecting the nuclear splicing machinery and assembly of virions (111). Recent studies in DUOX2 knockdown primary human nasal epithelial cells and in Duox2 inactivated mutant mice linked this oxidase isoform further to IAV host defense (154). Nox1 has also been connected to host protection in IAV infection as Nox1 deficiency resulted in increased chemokine and proinflammatory cytokine generation, and extensive 3-nitrotyrosine modification of murine lung tissue (155). In IAV infected mouse macrophages Nox2-derived endosomal ROS caused suppression of TLR7-dependent antiviral cytokines, suggesting distinct roles of phagocyte Nox2 (i.e. detrimental) in contrast to a beneficial role of Nox1 during IAV infection (156).

Pathogen recognition by epithelial cells induces chemokine production, leading to the recruitment of neutrophils and macrophages to the site of injury. Innate immune cells engage in proteolytic and oxidative killing of pathogens, mainly mediated by secondary reactive species such as hypochlorous acid and peroxynitrite that rely on NOX2, mitochondria, myeloperoxidase and iNOS (157–159). Superoxide generated by NOX2 is essential for host protection against pathogens, as mutational inactivation of NOX2 complex subunits, a characteristic feature of CGD patients, profoundly impedes the ability to clear certain bacterial and fungal infections (160). Studies using murine CGD hosts have advanced our understanding of the mechanisms associated with host protection by Nox2 that seem to depend on the pathogen and the microenvironment encountered. For instance, intestinal colonization by *Salmonella* Typhimurium was increased in streptomycin pretreated Nox2 deficient mice (i.e., *Cybb*$^{-/-}$) (161). Conversely, these mice were not more susceptible to infection with the murine pathogen *Citrobacter rodentium* (120). Superoxide produced by Nox2 played only a minor role in protection against *Mycobacterium tuberculosis* (Mtb) infection in contrast to reactive nitrogen species (RNS)-associated immunity, which seems preponderant in the control of murine lung infection with Mtb (162–165). Evasion of ROS-mediated killing (mROS and Nox2) was recently associated with Mtb virulence factor secretion and macrophage fatty acid catabolism, thereby improving mycobacterial survival in macrophages (166, 167).

These observations support the idea of a complex interaction between antimicrobial ROS, environmental factors, and virulence mechanisms employed by pathogens (e.g., detoxification enzymes), all impacting infection outcome and host protection (168). Redox-dependent mechanisms triggered by pathogen recognition in innate immune cells include the pathogen itself (e.g., size, type), and driving superoxide production in the appropriate subcellular compartment (e.g., phagosome, endosome, extracellular environment) by a specific source (e.g., NOX2, mitochondria) (169, 170). Upon pathogen uptake the phagosome is the main compartment where primary and secondary ROS accumulate in high concentrations. Assembly and activation of NOX2 at the phagosome membrane triggers the so-called 'phagocyte oxidative burst' that together with proteases supports killing of bacterial and fungal pathogens. In addition to phagosomal NOX2 activity, bactericidal ROS generation in the phagosome is maximized by mitochondria, with both ROS sources cooperating in pathogen killing (171). Mitochondria are recruited to the phagosome of infected macrophages, where they deliver mitochondria-derived effector molecules, including mROS in mitochondria-derived vesicles, to support intraphagosomal killing of pathogens (68, 172–174). Mitochondria mobility to the phagosome is promoted by the protein kinases Mst1/2. Upon phagocytosis, TLR-mediated signaling activates Mst1/2, triggering a TRAF6-Rac signaling axis involved in cytoskeletal rearrangements (172). The NOX2 complex localizes also to the plasma membrane, where it releases superoxide into the extracellular space in response to microbial patterns or large microorganisms that cannot be classically engulfed by phagocytic cells.

Neutrophils employ as additional host defense mechanism the release of nuclear chromatin, forming neutrophil extracellular traps (NETs). NETs act as defense mechanism against pathogens *via* entrapment and antimicrobial activity (175, 176). The sequence of events for NET formation is not completely defined (177), but it appears that NET release can be triggered in a ROS dependent or independent manner according to the pathogen or stimulus encountered (178–180). Neutrophils derived from CGD patients show an impairment in NET formation and ineffective control of *Aspergillus* species in the airways, leading to invasive aspergillosis. The importance of a functional NOX2 complex in restricting *A. nidulans* conidia and hyphae by NET formation was confirmed in a CGD patient undergoing gene therapy (181). The real-life situation of the severely compromised host immunity in CGD emphasizes the importance of ROS in host defense and serves as a reminder that certain disorders or disturbed mucosal environments may greatly benefit from improving oxidant production.

## IMPROVING ROS LEVELS AS THERAPEUTIC INTERVENTION

In pathological conditions ROS have been linked to 'oxidative stress' for decades. Oxidative stress is often retrospectively inferred when observing oxidatively modified proteins, lipids and DNA, yet without definite identification of the ROS generating enzyme(s) involved or how the damaging sequence of events was initiated. Moreover, several disease-causing genetic variants and disease-associated genetic risk factors have shed light on the harmful consequences of insufficient ROS production for $H_2O_2$-induced oxidative modifications, redox signaling and host defense (e.g., CGD, hypothyroidism, inflammatory bowel disease) (33). To date most strategies targeting oxidative stress (i.e., antioxidant therapy) are based on the delivery of ROS scavengers, ROS converting or degrading compounds or on non-selective enzyme inhibitors to protect tissues and organs, but clinical results of antioxidant treatments in inflammatory diseases have so far been disappointing (182–184). More recently, treatments aimed at generating excessive concentrations of ROS or at inhibiting cellular antioxidant defense systems at a designated target location (i.e., prooxidant therapy) have been developed to promote cancer cell death or antimicrobial killing through oxidative damage (185, 186). In contrast, ROS/redox-modulating therapies take a different, nontraditional approach. The goal of this strategy is a middle path intended to restore physiological levels of primary ROS (superoxide, $H_2O_2$) or, in certain circumstances, to moderately augment beneficial oxidants. This approach will require innovations in pharmaceutical drug technology such as nanotechnology and smart materials (e.g., liposomes, hydrogels, nanocarriers) enabling controlled delivery of therapeutic agents (e.g., ROS producing or converting systems) to a target site due to utilization of stimulus-sensitive materials (187–190). In some

settings ROS deficiencies may be partially compensated by pharmacological manipulation of endogenous oxidant producing enzymes. While still in their infancy science-based fine-tuning and balancing oxidant therapies are poised to deliver prophylactic and remedial benefits, namely improving homeostasis and host defense. This is particularly relevant for primary ROS with their essential role in redox signaling and dedicated immune functions. Here we summarize concepts for restoring physiological levels of beneficial oxygen-derived species in terms of technological approaches and therapeutic applications (**Figure 1**).

## Established and Emerging Strategies for Oxidant Generation

### Use of Hydrogen Peroxide

In the past, external application of $H_2O_2$ has been used for medicinal purposes (191, 192). Although high concentrations of $H_2O_2$ have well-known antiseptic properties, tissue damage may occur. On the other hand, application of lower concentrations of $H_2O_2$ (0.15-1.25 µmoles/wound) improved the rate of wound closure in wildtype mice and in mice deficient in Nox2-derived superoxide, thereby accelerating tissue regeneration (193, 194). Topical application of micromolar $H_2O_2$ promoted the phosphorylation of tyrosine residues in FAK that supported an angiogenic response *via* vascular endothelial growth factor (VEGF) signaling. Removal of $H_2O_2$ by addition of the $H_2O_2$ degrading enzyme catalase delayed wound healing and impeded angiogenesis (193). While these experiments may suggest direct exposure of skin to $H_2O_2$ as wound healing treatment, the therapeutic window is too narrow for safe and efficient tissue restitution. Further, internal administration of $H_2O_2$ solutions poses serious health risks and should not be promoted. To achieve a health benefit, technologies permitting modified release of a standardized dose of nanomolar $H_2O_2$ to specific target areas will be required. Strategies promoting controlled $H_2O_2$ delivery may include the utilization of nano systems, diffusible molecules, and stimulus-sensitive compounds (195–198). Stimulus-responsive materials are particularly attractive to target specific disease-associated microenvironments such as changes in pH due to inflammation (189, 199, 200). In accord, one can imagine the development of redox-sensitive materials actively releasing appropriate oxidants until a certain threshold is

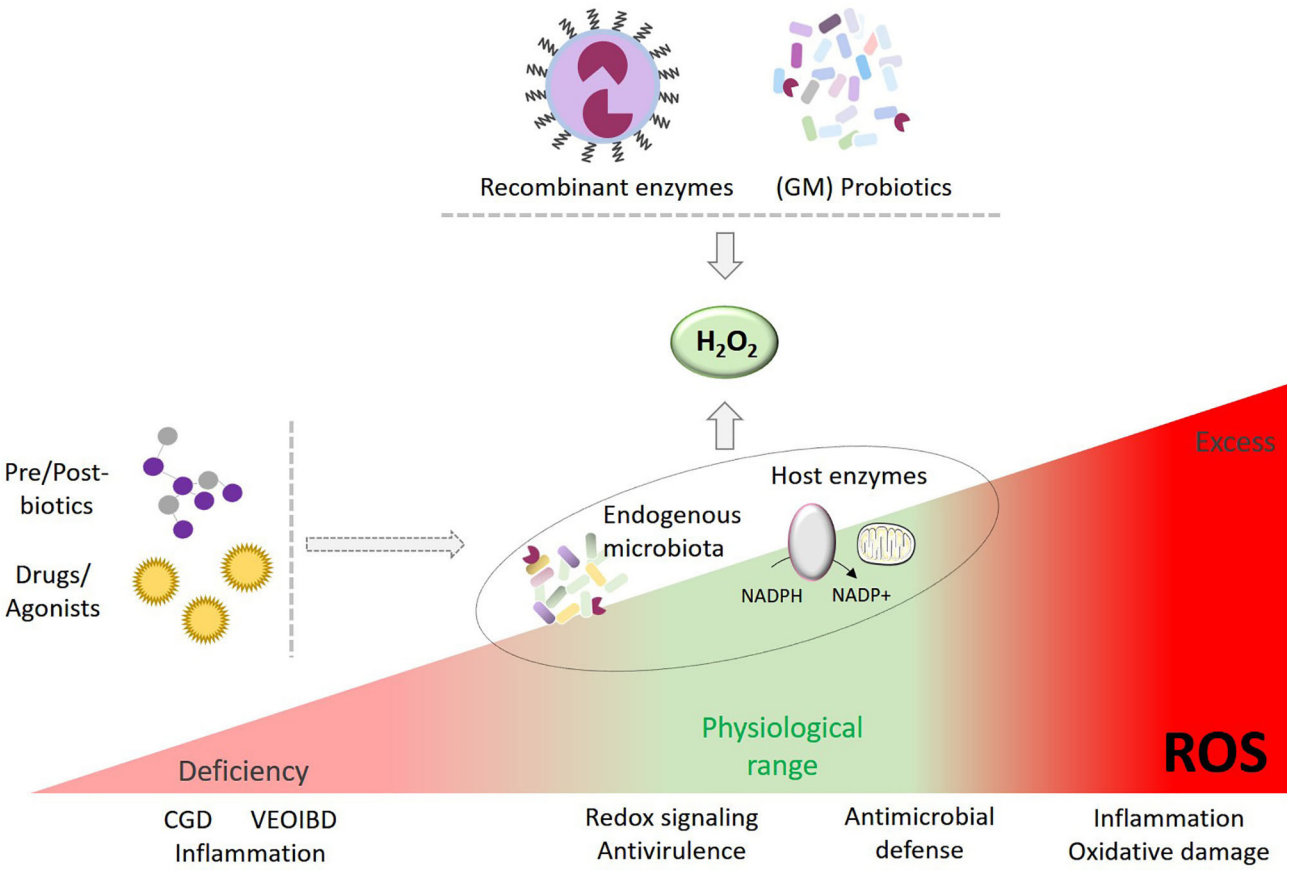


**FIGURE 1** | Enhancing $H_2O_2$ as therapeutic intervention. Strategies to restore or improve ROS in a controlled manner may include a) administration of selected probiotics or genetically-modified (GM) bacteria producing $H_2O_2$, b) innovative drug technology delivering recombinant $H_2O_2$ generators to a target area, c) pre-/post-biotics and d) drugs and agonists modulating ROS-generating host enzymes or endogenous microbiota to augment $H_2O_2$ production.

achieved and the system self-inactivates. Moreover, the vehicle strategy is important. For instance, hydrogels are commonly used as drug carriers for clinical use, owing to their tunable mechanistic and physicochemical properties (201). Controlled and continuous release of $H_2O_2$ was achieved with an *in situ* forming hydrogel that can be used as antimicrobial wound dressing (202).

## Lactobacillus as $H_2O_2$ Source

Lactobacilli, widely used probiotic bacteria that belong to the gastrointestinal and vaginal microbiota (203), are the microbial prototype for controlled release of nanomolar $H_2O_2$. Many *Lactobacillus* strains utilize oxygen to generate $H_2O_2$ by various enzymes including flavin reductase (nfr), NADH oxidase (nox) or pyruvate oxidase (pox) (204–207). Lactobacilli colonizing the mucus layer are close enough to the oxygen gradient emanating from host epithelial barrier cells (up to 3% $O_2$) to secrete nanomolar $H_2O_2$ continually (208–210). A recent report linked *Lactobacillus* generated $H_2O_2$ to increased colonization resistance, protecting the host from *C. rodentium* infection (120). A decline in host-derived $H_2O_2$ due to genetic deletion or loss-of-function mutations in NADPH oxidases gives rise to microbiota dysbiosis, which is likely niche-driven and tied to changes in the intestinal microenvironment. Thus, manipulation of the microbiota with lactobacilli constitutes a potential strategy to promote microbiota diversity for improved colonization resistance. This approach can be extended to the airways colonized by their unique microbiota. Intranasal administration of probiotics (including various *Lactobacillus* strains) was recently proposed to modulate local immunity and epithelial barrier function (211, 212). In addition to living lactobacilli, administration of prebiotics to selectively stimulate the growth or activity of resident lactobacilli may improve the barrier redox environment and stimulate host immunity. For example, abundance of lactobacilli seems to increase following the consumption of galacto-oligosaccharides, a common prebiotic generated from the decomposition of lactose, with reported beneficial effects on immune function (203, 213).

Early findings that $H_2O_2$ promotes cell migration match with the positive effects of lactobacilli on wound healing. Lactobacilli secrete not only $H_2O_2$ but numerous other compounds such as lactic acid and bacteriocins that improve the wound environment. Nevertheless, the ability of *L. johnsonii* to generate $H_2O_2$ was directly linked to accelerated recovery and tissue restitution in murine colitis (210). Transformation of lactobacilli to express exogenous proteins can further improve their wound healing capacity as demonstrated recently for *L. reuteri* secreting CXCL12 (214). Lactobacilli may also release yet undefined compounds that trigger intracellular ROS generation by host cells, for example by the oxidase Nox1, to improve mucosal repair (85, 105). Bacteria or microbial-derived products can drive expression of ROS-generating enzymes in the host, likely associated with increased $H_2O_2$ levels. Ileal colonization with commensal segmented filamentous bacteria (SFB) increased expression of the oxidase Duox2 (19). Intraperitoneal administration of SFB-derived flagellin was sufficient to upregulate Duox2 in the small intestine, suggesting that microbial regulation of Duox2 expression may be TLR5 dependent (215).

Most studies linking lactobacilli to antimicrobial defense, mucosal healing and microbiota modification have been conducted in mice. In clinical trials the outcome of probiotic therapy in IBD has been mixed (216–219). Possible explanations range from poor standardization and viability of bacteria to insufficient colonization due to the human intestinal environment including the mucus layer, as well as to differences between mouse and human physiology such as the presence of a *Lactobacillus* reservoir in the murine forestomach. Another confining factor is using living organisms, which can lead to systemic bacterial dissemination. Several reports outline the risk for *Lactobacillus* bacteremia and septicemia in immunocompromised patients, after surgical procedures and in colitis patients (220–223). Even in mice septicemia was observed when either the orally administered dose of *L. johnsonii* was increased ten-fold or *L. johnsonii* overproducing $H_2O_2$ was administered, indicating that $H_2O_2$ production should not exceed an optimal physiological range for health benefit (210). Current developments in the field are focused on standardization, design of consortia and recombinant probiotics, but other inventive strategies as outlined below could address some shortcomings of probiotics as $H_2O_2$ generators.

## Application of Recombinant Enzymes

Recombinant enzymes involved in ROS generation or conversion can be used to modulate the oxidative microenvironment. The enzyme superoxide dismutase (SOD) catalyzes the dismutation of superoxide into oxygen and $H_2O_2$. Several isoforms of this enzyme are expressed in both mammalian and bacterial hosts, where they serve as part of the protective antioxidant system. SOD-mediated superoxide conversion limits the generation of secondary, highly reactive oxygen and nitrogen metabolites (e.g., hydroxyl radical, peroxynitrite) that can cause irreversible modification of proteins, lipids or DNA (224, 225). Therefore, SOD-based applications have been mainly used as antioxidant strategy aiming to decrease secondary ROS levels for prevention of inflammatory diseases. A more universal utilization of SOD as physiological $H_2O_2$ source at mucosal surfaces is compromised by its mode of action, including reliance on inflamed conditions, in conjunction with unattainable dosing standardization, and poor enzymatic stability (226).

Many oxidoreductases generate $H_2O_2$ as a byproduct of their intended enzymatic reaction including carbohydrate oxidases [e.g., glucose oxidase, galactose oxidase and many others (227)], cholesterol oxidase (228), alcohol oxidase (229) and D-amino acid oxidase (230). We will discuss here as example glucose oxidase (GOx), a prominent representative of carbohydrate oxidases that catalyzes the oxidation of β-D-glucose to D-glucono-1,5-lactone with further hydrolysis to gluconic acid. GOx contains the flavin adenine dinucleotide (FAD) cofactor as initial electron acceptor, which undergoes reduction to FADH2, followed by FADH2 oxidation by molecular oxygen, and reduction of oxygen to $H_2O_2$ (231). GOx is found

predominantly in fungi (i.e., *Aspergillus* and *Penicillium* spp) and its high specificity for glucose renders the enzymatic reaction an attractive tool for monitoring blood glucose levels. Various GOx-based biosensors have been developed for glucose monitoring, sometimes in diabetes therapy in conjunction with insulin release (232, 233). Stability of the enzyme is important for these applications, and thus technology advancements have resulted in genetically engineered GOx modifications in combination with encapsulation strategies for improved long-term enzymatic activity (234). Dermatological applications of GOx as tunable $H_2O_2$ generator may hold some promise for therapy. Incorporation of GOx in a collagen dressing improved wound healing and tissue regeneration of rodent skin, presumably due to sustained release of $H_2O_2$, although oxidant measurements were not provided. This GOx treatment induced an antioxidant response by the host tissue with upregulation of SOD and catalase (235). GOx is naturally present in honey at low concentrations where it has been studied for its dual function, namely for aiding wound healing and as antimicrobial agent. A medicinal honey (SurgihoneyRO$^{TM}$) with enhanced antimicrobial activity is on the market as a wound antiseptic dressing (236, 237). Due to its GRAS (generally recognized as safe) FDA status GOx is used by the food industry as stabilizing and antibacterial agent (238).

The GRAS status supports considering GOx for internal applications such as hydrogels or oral administration of the enzyme. A gelatin hydrogel incorporating glucose and varying GOx concentrations released micromolar $H_2O_2$ for 24-48 hours in a controlled manner and improved the proliferation of cultured endothelial cells. This effect was accompanied by a transient increase of intracellular ROS (measured as DCFH-DA signal) as well as enhanced neovascularization in a CAM model, indicating modulation of redox signaling by GOx-generated $H_2O_2$ (239, 240). For oral administration, the protection of GOx's enzymatic activity can be achieved by microencapsulation. Diet delivered glucose might be sufficient for GOx-mediated $H_2O_2$ generation in the small intestine, while supplementation of the GOx drug carrier with compartmentalized glucose will be necessary for affecting the large intestine. Targeted, controlled delivery of GOx/glucose presents a promising opportunity to modulate intestinal redox signaling, mucosal healing and immune defense while limiting oxidative damage. Therapeutic applications of GOx/glucose or similar $H_2O_2$ generators will partially mimic the current use of lactobacilli, albeit with superior standardization, more limited dependence on the barrier microenvironment and superior safety in vulnerable patient populations. An additional benefit of the GOx/glucose reaction is the generation of the prebiotic gluconic acid as a secondary reaction product, which stimulates butyrate production in the intestine (241). Examples for potential treatment modalities are gut health improvements (e.g., barrier reinforcement, microbiota diversity), accelerated tissue restitution after injury in patients with intestinal inflammatory diseases, or prophylactic long-term modification of the intestinal environment in patients with insufficient $H_2O_2$ generation. Prime examples for the last point are certain patient populations in the categories very early onset IBD (VEO-IBD), CGD-IBD or IBD linked to variants upstream of ROS-generating enzymes (21, 242–244). Variants in genes encoding components of NOX enzymes including NOX1, NOX2 and DUOX2 confer susceptibility to VEO-IBD (152, 245–248), while NOX2 complex variants associated with CGD due to absent or minimal output of superoxide manifest in 40-50% of CGD patients as CGD-associated IBD. Functional evaluation of NOX1 and DUOX2 patient variants in model systems revealed decreased ROS production and impaired antimicrobial defense (152, 246, 247). The compromised immune defense of these hosts in combination with the ensuing dysbiotic microbiota affects not only the overall colonization resistance but also more specialized immune defense mechanisms. Diffusion of nanomolar $H_2O_2$ into extracellular pathogens is not bactericidal but can downregulate virulence factors. Nanomolar $H_2O_2$ blocked bacterial phosphotyrosine signaling required for virulence factor synthesis by irreversibly modifying tyrosine phosphorylated enzymes and proteins (22, 23). In addition, nanomolar $H_2O_2$ inhibited LEE pathogenicity island regulation in enteropathogenic bacteria (*C. rodentium*) due to impeded expression of the major transcriptional regulator *ler*, which was associated with lower pathogen colonization and improved recovery of mice (120). While the precise molecular mechanism of LEE downregulation has not yet been resolved, these studies hold great promise for $H_2O_2$-mediated interference in enteric bacterial infections. Supplying nanomolar levels of $H_2O_2$ *via* GOx/glucose or similar means may provide multiple benefits including host protection, reinforcement of the intestinal barrier and mucosal healing.

## Stimulation of Endogenous ROS Sources

Instead of providing ROS by an exogenous source, agonists or drugs that stimulate expression and/or activation of a ROS-generating enzyme or alter mitochondrial function can be used to improve immune responses. Currently this area is dominated by improving innate immune cell-derived superoxide, but one can envision that future applications will be directed at epithelial superoxide/$H_2O_2$-producing enzymes. Neutrophils and macrophages are crucial for pathogen defense with NOX2-derived superoxide playing a key role in this process. Enhancing the neutrophil activation status, and in particular the oxidative burst, will not only be desirable in infections but also in inflammatory disorders (249–251). Activating NOX2 can be achieved by targeting upstream pathways. One approach focusses on developing formyl peptide receptor (FPR1, FPR2) agonists selectively binding to a receptor conformation that will favor one signaling output over another (i.e. biased signaling) without accelerating receptor desensitization (249). In neutrophils FPRs regulate directional migration, secretion of inflammatory mediators and superoxide generation by NOX2. While FPRs are usually triggered by microbial derived formylated peptides, endogenous proteins or lipopeptides, small compounds and peptides acting as exogenous FPR agonists have been identified. Examples are the FPR1 agonist RE-04-001, FPR2 agonists BMS-986235 and Act-389949, and dual FPR1/2 agonists such as compound 17b (252–255).

Biased FPR agonists causing functional selectivity in neutrophil responses, namely either inducing chemotaxis or superoxide production, can be used as NOX2 activators (252, 256). Nox2 activity has been associated with the resolution of inflammation in colitis (133), and the pro-resolving Fpr2 agonist Annexin-A1 accelerated wound healing of the murine colonic epithelium *via* the oxidase Nox1 (81).

Another approach is using the cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) to augment NOX2 derived superoxide production by acting as priming agent in neutrophils while elevating the phagosomal pH in macrophages (257–259). The efficacy of GM-CSF administration as immunostimulatory adjuvant has been evaluated in clinical trials. In a randomized trial involving critically ill patients, subcutaneous injections with GM-CSF increased neutrophil phagocytic capacity but did not improve superoxide production (EudraCT 2011-005815-10), while an earlier trial using intravenous delivery of GM-CSF had reported statistically significant enhancement of neutrophil superoxide generation (260). GM-CSF has also been assessed in the context of CGD patients. While reconstitution of the oxidative burst in isolated neutrophils was not achieved, as expected for a disease caused by genetic mutations, GM-CSF has shown some promise in treating CGD-IBD by undefined mechanism(s) (261–263). In non-CGD patients biased targeting of NOX2 activating pathways may hold promise, and analogous strategies should be feasible for development of activators of other NOX/DUOX enzymes or of other ROS sources.

### Generation of Compensatory ROS

Therapeutically enhancing ROS production in CGD or VEO-IBD patients is imperative for antimicrobial defense, for resolution of inflammation and for repair processes. In these patients gene editing, administration of exogenous ROS generators or stimulation of other endogenous ROS sources are the only options to improve their ROS status. Kuhns and coworkers showed that modest residual superoxide production is sufficient to prevent life-threatening infections in CGD patients (264). Partial restoration of macrophage and dendritic cell superoxide in functional *Ncf1* rescue mice was adequate to dampen hyperinflammation (265). These considerations are the basis for screening and identifying compounds capable of compensating partially for NOX2 enzyme activity.

The first compound in this class is the peroxisome proliferator-activated receptor γ (PPARγ) agonist pioglitazone, a drug approved for Type 2 diabetes. As proof of concept, pioglitazone treatment of cultured CGD patient-derived monocytes or of Nox2 deficient mice increased mROS production and subsequently the bactericidal capacity of immune cells (266). Pioglitazone and the related rosiglitazone induced mROS and NETs in CGD neutrophils (267). Monocytes from CGD patients and Nox2 deficient macrophages were impaired in PPARγ signaling, impacting their efferocytosis function which was restored by pioglitazone treatment (268, 269). Increased mROS generation and restoration of efferocytosis were also observed in monocytes isolated from two CGD patients after 30 days of oral pioglitazone treatment, supporting the effectiveness of pioglitazone therapy for superoxide production and improved immune cell function (269). A clinical study reported the effects of daily pioglitazone treatment in an infant with CGD. Using dihydrorhodamine (DHR) fluorescence as readout for ROS an increase in DHR positive granulocytes after 25 days of treatment was observed. The increase in the phorbol ester stimulated DHR signal was relatively low in comparison with healthy donor cells but it was maintained over several weeks, and the clinical condition of the patient progressively improved (270). Efficacy and safety of pioglitazone was assessed in a clinical trial enrolling CGD patients with severe infection. Phase 2 of the study was terminated when the DHR fluorescence signal did not improve in neutrophils after 90 days of treatment with pioglitazone (clinicaltrials.gov NCT03080480). Long-term administration of pioglitazone did not lead to drug-related adverse effects or exacerbation of infection (271). The variation in outcomes might be attributable to different pioglitazone dosage, or to the often required combination therapy with antibiotics and/or interferon IFN-γ. Further studies are necessary to determine if pioglitazone will provide a therapeutic option for CGD patients, for example as adjuvant therapy in severe bacterial infections or as prophylaxis. At this point only hematopoietic stem cell transplantation, gene therapy or gene editing with CRISPR-Cas9 offer a cure for CGD (272).

## Future Directions

The field of redox medicine has blossomed over the last decade, but further progress will depend on connecting more tightly distinct oxidant species and their enzymatic sources to physiological or pathophysiological processes. Another important factor will be uncovering and manipulating specific microenvironments. The chemical milieu, in particular oxygen availability, is a limiting factor for ROS production, but the hypoxia developing due to ROS production (e.g., *via* the neutrophil oxidative burst) in a low oxygen environment can have beneficial effects such as resolution of inflammation and accelerated tissue restitution (133). The presence and concentration of $H_2O_2$ and secondary oxidants (hypochlorous acid, peroxynitrite) will modify the barrier environment and the interactions between host and microbiota, leading to changes in microbiota diversity, composition, and the microbial metabolome that can be beneficial or harmful for the host. Case in point is the intestinal dysbiosis and appearance of pathobionts in NADPH oxidase deficient mice (94, 113). The nanomolar $H_2O_2$ released from mucosal barriers is inadequate for bactericidal activity, but its interference with the fitness and virulence of certain microorganisms supports its use as a promising alternative to antibiotics (273, 274). However, this antivirulence strategy will require the identification and targeting of specific pathogens to avoid the subversion of these oxidants for their own advantage as reported for some pathogenic bacteria (275–281).

Given the essential role of ROS for basic physiological functions, future therapeutics should include careful modulation of the redox state. Therapeutic interest today focuses on the margins of the ROS continuum, namely anti- or

pro-oxidant interventions, but new approaches to modify physiological redox processes will be vital for improving health and well-being. Current limitations are the lack of targeted drugs modulating the activity of specific enzyme isoforms or of drugs that can adapt their mode of action by sensing ROS levels in situ. Sophisticated delivery systems for novel small molecule- or peptide-based activators will help restoring or enhancing beneficial ROS (mainly $H_2O_2$) at a predetermined location in order to achieve a positive outcome and to prevent undesired side effects linked to toxicity. For this purpose, exploitation of natural properties of commensals or their genetic modification, discovery of agonists stimulating ROS-generating enzymes, design of functional materials and delivery vehicles releasing $H_2O_2$, for example by utilizing recombinant enzyme-substrate pairs, will provide therapeutic options for infections, inflammatory diseases, and regenerative medicine. Next generation treatments will likely include strategies for altering the chemical environment at defined locations in a sustainable manner, with ROS being one of the key targets.

## AUTHOR CONTRIBUTIONS

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# Targeting Mitochondrial-Derived Reactive Oxygen Species in T Cell-Mediated Autoimmune Diseases

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Mitochondrial dysfunction resulting in oxidative stress could be associated with tissue and cell damage common in many T cell-mediated autoimmune diseases. Autoreactive CD4 T cell effector subsets (Th1,Th17) driving these diseases require increased glycolytic metabolism to upregulate key transcription factors (TF) like T-bet and RORγt that drive differentiation and proinflammatory responses. However, research in immunometabolism has demonstrated that mitochondrial-derived reactive oxygen species (ROS) act as signaling molecules contributing to T cell fate and function. Eliminating autoreactive T cells by targeting glycolysis or ROS production is a potential strategy to inhibit autoreactive T cell activation without compromising systemic immune function. Additionally, increasing self-tolerance by promoting functional immunosuppressive CD4 T regulatory (Treg) cells is another alternative therapeutic for autoimmune disease. Tregs require increased ROS and oxidative phosphorylation (OxPhos) for Foxp3 TF expression, differentiation, and anti-inflammatory IL-10 cytokine synthesis. Decreasing glycolytic activity or increasing glutathione and superoxide dismutase antioxidant activity can also be beneficial in inhibiting cytotoxic CD8 T cell effector responses. Current treatment options for T cell-mediated autoimmune diseases such as Type 1 diabetes (T1D), multiple sclerosis (MS), rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE) include global immunosuppression, antibodies to deplete immune cells, and anti-cytokine therapy. While effective in diminishing autoreactive T cells, they can also compromise other immune responses resulting in increased susceptibility to other diseases and complications. The impact of mitochondrial-derived ROS and immunometabolism reprogramming in autoreactive T cell differentiation could be a potential target for T cell-mediated autoimmune diseases. Exploiting these pathways may delay autoimmune responses in T1D.

**Keywords: autoimmunity, T cell, immunometabolism, mitochondria, reactive oxygen species**

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## INTRODUCTION

Failure to maintain self-tolerance leads to autoreactive T cells that recognize systemic or organ-specific self-antigens and subsequently, the development of autoimmunity (1). Mechanisms that result in decreased regulatory T cell (Treg) numbers and/or function could be contributing to self-tolerance failure. Tregs are essential in maintaining self-tolerance by secreting immunosuppressive/anti-inflammatory cytokines including IL-10 and TGF-β, and expression of the inhibitory receptor, CTLA-4 (2, 3) (**Figure 1**). Re-establishing self-tolerance by increasing Treg cell numbers and/or increasing Treg immune suppression function may inhibit autoreactive T cell effector responses and delay the progression of T cell-mediated autoimmune diseases (4). CD4 T cell subsets associated with T cell-mediated autoimmune diseases include T helper (Th) Th17 and Th1 cells.

Generally, Th17 cells mediate immunity to extracellular pathogens and are characterized by the expression of the transcription factor (TF) RORγt and the production of cytokines such as interleukin (IL)-17A/F (5) (**Figure 1**). Th17 cells are involved in autoimmune-mediated diseases such as multiple sclerosis (MS), rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE) (6, 7) (**Figure 1**). Th1 T cell responses are responsible for cell-mediated immunity and are characterized by the expression of the TF T-bet and the production of IFN-γ (8) (**Figure 1**). In Type 1 diabetes (T1D), CD4 T cell Th1 cytokine responses and CD8 cytotoxic T cell responses contribute to inflammation and destruction of insulin-producing β-cells (9) (**Figure 1**).

Current strategies to treat T cell-mediated autoimmune diseases include the use of low dose IL-2, mTOR inhibition (rapamycin), T cell depletion antibodies (teplizumab, anti-thymocyte globulin), or cytokine neutralizing antibodies/soluble receptor proteins (Infliximab, Etanercept) (10–12). These treatments are successful in temporarily diminishing disease pathogenesis; however, these reagents do not provide a permanent cure for autoimmune diseases. Therefore, further research is required to fully understand the mechanisms involved in autoreactive T cell differentiation in autoimmune disease development. One mechanism that is involved in T cell fate and function includes cellular metabolism and specifically, the interplay between signaling pathways involved in T cell differentiation and metabolic reprogramming to determine T cell effector responses (13, 14).

Immunometabolism has garnered extensive attention in recent years due to the reliance of specific metabolic pathways

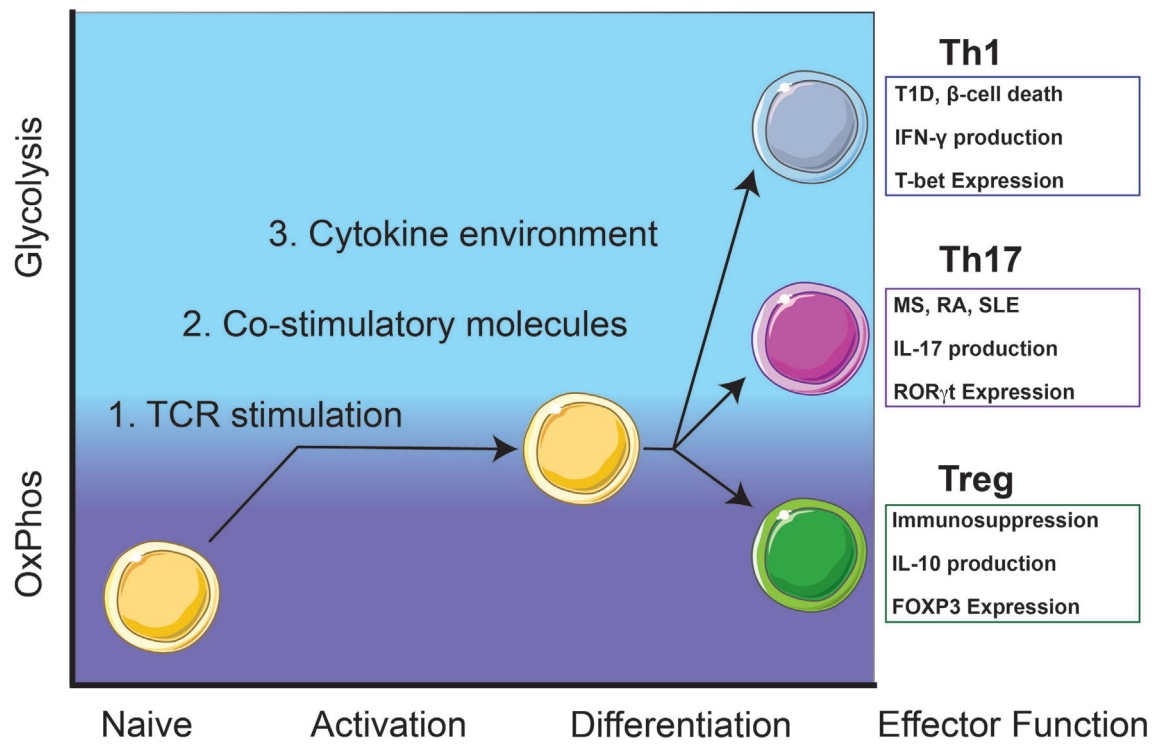


**FIGURE 1 | Autoreactive Th1 and Th17 T cell responses rely on glycolysis while immunosuppressive Treg cells utilize oxidative phosphorylation (OxPhos).** Th1 cells contribute to β-cell death and destruction in type 1 diabetes (T1D) and Th17 cells contribute to pathogenesis of other T-cell mediated diseases including multiple sclerosis (MS), rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE). Upon activation, naïve T cells will metabolically shift from OxPhos-dependence to a balance between glycolysis and OxPhos. Throughout early activation and differentiation, this balance is maintained until a commitment toward an effector function is achieved. Once fully differentiated, autoreactive Th1 and Th17 cells utilize glycolysis for homeostasis and maintenance while immunosuppressive Treg cells rely on OxPhos.

necessary for efficient T cell activation and differentiation of T cell subsets. Immunometabolic pathways of interest includes glycolysis, oxidative phosphorylation (OxPhos), and the contribution of mitochondrial-derived reactive oxygen species (mtROS) to mediate autoreactive T cell activation and differentiation by functioning as signaling molecules (7, 15, 16). Therefore, reprogramming T cell metabolism by targeting mtROS may counteract autoreactive T cell function and proinflammatory responses (17). This review will discuss the impact of mtROS and its potential target for immunometabolism reprogramming therapy in the context of T cell-mediated autoimmune diseases.

## ACTIVATION OF NAÏVE CD4 T CELLS ARE PRIMED FOR DIFFERENTIATION BY SHIFTING METABOLISM AWAY FROM OXIDATIVE PHOSPHORYLATION

Initially, metabolically quiescent naïve CD4 T cells rely on OxPhos to meet all their metabolic needs (18) (**Figure 1**). During activation, T cells undergo a shift in metabolism away from OxPhos by increasing glycolysis. T cell activation requires three main signals from antigen-presenting cells (APC); antigen presentation on major histocompatibility complex (MHC), co-stimulation, and cytokines/reactive oxygen species (ROS) (16, 19). CD4 T cell activation begins with an interaction between the T cell receptor (TCR) and peptide loaded on MHC class-II which initiates metabolic reprograming on naïve CD4 T cells (20). Co-stimulatory receptors present on both APCs and T cells will provide the second signal necessary for T cell activation. Finally, cytokines and ROS present in the local environment will provide a proinflammatory third signal to begin T cell differentiation toward specific effector responses (16, 21). Each signal lays the foundation for potential T cell fates and drives the shift in metabolism.

TCR stimulation increases hydrogen peroxide signaling to support the upregulation of interleukin (IL)-2 (22, 23). The TCR is comprised of α and β chains and CD3 accessory chains that cumulatively bridge extracellular antigen presentation by APCs to facilitate into intracellular signaling (19, 24). After TCR stimulation, there is an influx of CD3-dependant store-operated $Ca^{2+}$ entry (SOCE) that induces the accumulation of mitochondria near the TCR (23) (**Figures 2A, B**). $Ca^{2+}$ influx into localized mitochondria influences IL-2 secretion by impacting the production of hydrogen peroxide (22) (**Figures 2C, D**). Due to its ability to stimulate differentiation and proliferation of T cells, production of IL-2 is a key cytokine for T cell activation (24). Deficiency in SOCE channels, such as $Ca^{2+}$ release-activated $Ca^{2+}$, results in a lack of IL-2 production that is potentially due to decreased hydrogen peroxide synthesis (25).

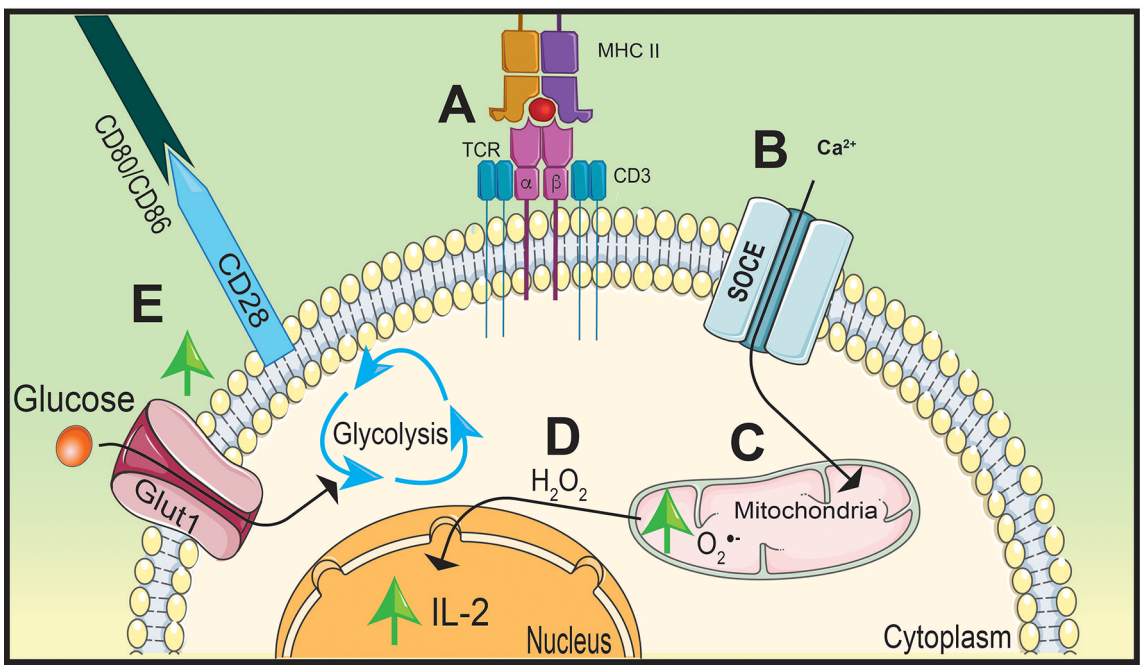


**FIGURE 2** | **Stimulation of TCR and CD28 causes a metabolic shift in naïve CD4 T cells.** T cell receptor (TCR) stimulation by peptide presented on major histocompatibility complex-II (MHC-II) **(A)** will increase calcium ($Ca^{2+}$) entry into the cytoplasm through store operated calcium entry (SOCE) channels **(B)** increasing mitochondrial-derived superoxide ($O_2^{\bullet-}$) generation **(C)**. Oxidative phosphorylation (OxPhos) will promote interleukin (IL)-2 expression by hydrogen peroxide ($H_2O_2$) signaling **(D)**. Simultaneously, CD28 will upregulate Glut1 expression on the cell membrane increasing glucose uptake shifting metabolism away from OxPhos to glycolysis to support rapid proliferation **(E)**.

While $Ca^{2+}$ influx is important to promote IL-2 production, the essential signaling molecule is hydrogen peroxide. This concept is supported by $Ca^{2+}$-independent nuclear factor kappa-B (NF-κB) signaling, shown to be activated by hydrogen peroxide, significantly inhibiting IL-2 promoter regions as a result of blocking $Ca^{2+}$ and/or ROS synthesis (26, 27). Similar to NF-κB signaling, hydrogen peroxide also mediates nuclear factor of activated T cells (NFAT) nuclear translocation and interaction with AP-1 to induce IL-2 production (28). These reports highlight the importance of $Ca^{2+}$ influx and mtROS production to facilitate IL-2 production and T cell activation. However, optimal T cell activation also requires co-stimulatory molecule interactions in order to induce multiple signaling pathways and shift metabolism away from OxPhos to initiate T cell effector differentiation (29).

Co-stimulatory molecule, CD28, will increase glycolytic activity within the T cell to promote rapid proliferation (30, 31). Engagement of the TCR with the MHC-II/peptide complex forms an immunological synapse between the APC and the CD4 T cell by recruiting co-stimulatory molecules organized on lipid rafts on the cell membrane (32). This includes CD28 on the T cell which will interact with CD80 and CD86 on the APC (19). In order to maximize glycolytic potential, activated T cells will upregulate glucose transporter 1 (Glut1) to the cell membrane following co-stimulation and engage in aerobic glycolysis to ensure glucose will be utilized despite the presence or availability of oxygen (33, 34) (**Figure 2E**). CD28 will also upregulate signaling pathways that support T cell differentiation and glycolysis (31). One key pathway that is essential for T cell activation and proliferation is mitogen-activated protein kinase (MAPK) (30).

Within this pathway, extracellular signal-regulated kinase (ERK) signaling can regulate cell proliferation and differentiation by promoting glycolysis, facilitating the switch from OxPhos (35). While TCR stimulation will initiate ERK signaling in T cells, CD28 influences ERK activation by recruiting the appropriate kinases, such as lymphocyte-specific protein tyrosine kinase to enhance the signaling cascade (31). ERK phosphorylation in naïve T cells negatively regulates TCR-induced $Ca^{2+}$ influx reducing OxPhos and promoting glycolysis (36). High glucose and glycolytic activity within the cell will support ERK/MAPK signaling, increase cell proliferation, differentiation, and prevent apoptosis (30, 37). The kinetics of T cell activation would be greatly diminished without CD28-dependent effects on ERK signaling and may result in weakened T cell responses (31).

In order to ensure a strong T cell response, CD28 stimulation also increases expression of pro-survival proteins including BCL-XL to prevent cell death in rapidly expanding T cells (38). Under non-apoptotic conditions, including T cell activation and expansion, overexpression of BCL-XL will result in mitochondrial depolarization and halting OxPhos (39). BCL-XL is able to influence OxPhos by reducing intracellular $Ca^{2+}$ channel type 1 inositol 1,4,5-triphosphate receptor, which decreases the magnitude and duration of $Ca^{2+}$ release following TCR signal transduction (39). BCL-XL will also redirect cytosolic $Ca^{2+}$ away from the mitochondria through ER sequestration (39). Once co-stimulation has induced a metabolic state that is balanced between OxPhos and glycolysis; cytokines and ROS present in the immune environment will prime activated T cells for differentiation into specific effector subsets and responses.

The efficacy of both TCR and co-stimulatory signaling is greatly influenced by cytokines and ROS present during T cell activation (16, 21). Cytokine signaling can function in a paracrine and autocrine manner to influence APC and T cell activation (40). Downstream signaling pathways such as mTOR, NFAT, and NF-κB involved in T cell activation and differentiation are not only altered by the concentration of cytokines, but also by their kinetic expression as well (13, 21). Prior to activation, IL-7 is an essential cytokine for naïve T cell survival and homeostasis due to its inactivation of pro-apoptotic proteins (41, 42). Upon stimulation, T cells will secrete IL-2 to promote expansion and prime T cells for differentiation (41). An autoreactive Th1 effector response will result from a cytokine environment comprised of IFN-γ, IL-1β, TNF-α, IL-6, and IL-12 (43). IL-17, IL-23, and IL-21 cytokines result in an autoreactive Th17 effector response; while TGF-β and IL-10 will result in the promotion of self-tolerance (43).

Traditionally it was thought that cytokines were solely responsible for driving T cell differentiation toward specific effector responses. However, superoxide may also be contributing to differentiation by activating redox-dependent signaling pathways associated with certain T cell effector functions (44). Past research has demonstrated that even under conditions which utilized cytokines to skew toward a proinflammatory response, the reduction of ROS results in decreased NF-κB-regulated proinflammatory cytokine production (45). Additionally, the decrease in ROS, specifically superoxide, in both APC and T cells not only decreased proinflammatory IFN-γ synthesis, but also T-bet expression as well (16). These data accentuate the indispensable role of ROS generation in effector T cell responses.

## MITOCHONDRIAL DYSFUNCTION IS COMMON THROUGHOUT T CELL MEDIATED DISEASES

Due to the role of ROS in cellular homeostasis, irregular ROS generation contributes to disease pathology making it a prime target for metabolic reprograming therapies (46). ROS including superoxide are mainly generated by the mitochondria, but may also be produced by NADPH oxidase enzymes (47, 48). Antioxidants present within the cell and extracellular environment regulate ROS to prevent damage to the cells caused by oxidative stress (49). Oxidative stress and mitochondrial dysfunction are common pathologies normally found in T cell-mediated autoimmune diseases (49, 50), indicating that increasing our understanding of oxidative stress and ROS regulation may have potential for the development of novel therapies to mitigate autoimmune dysfunction.

ROS are generated throughout the cell as a result of various redox reactions (47, 48). The mitochondria are one of the main cellular sources of ROS synthesis due to electrons leaking from the election transport chain (ETC) and interacting with oxygen to generate superoxide (47) (**Figure 3A**). Pyruvate generated from glycolysis will migrate into the mitochondrial matrix, be converted to acetyl-CoA, and feed into the citric acid cycle (CAC) (51). Succinate dehydrogenase, also known as complex II, is the only membrane-bound component of the CAC and a complex within the ETC (52). Electron movement creates a proton gradient between the mitochondrial matrix and the inner membrane space driving the phosphorylation of adenosine diphosphate to adenosine triphosphate (ATP) by ATP synthase (53). Superoxide generation is not limited to the mitochondria, it can also be generated by NADPH oxidase enzymes on the plasma membrane (48) (**Figure 3B**).

Excess levels of superoxide can be toxic to the cell; therefore, superoxide dismutase (SOD) enzymes act to dismutate superoxide into hydrogen peroxide and molecular oxygen (46). There are three different isoforms of SOD located within and outside the cell. Copper- and zinc-containing SOD (Cu/Zn SOD, SOD1) is mainly localized in the cytoplasm (54) (**Figure 3C**). Manganese-containing SOD (MnSOD, SOD2) resides within the mitochondrial matrix (54) (**Figure 3A**). Similar to SOD2, extracellular SOD (SOD3) also contains manganese, except it is localized outside the cell (54) (**Figure 3D**). Regardless of its location, enzymatic regulation of superoxide by SOD will generate hydrogen peroxide as a result of its reaction (48) (**Figure 3C**). Unlike superoxide, hydrogen peroxide is stable, diffusible, and can regulate redox-dependent signaling pathways (55) (**Figure 3E**). Hydrogen peroxide can modulate pathway activation by inhibiting phosphatases, activating tyrosine kinases, and transcription factor activation (55). Catalase, glutathione peroxidase, and thioredoxin peroxidase are the main enzymes involved in maintaining hydrogen peroxide levels for optimal cell function and signaling (56).

A common factor in several mitochondrial-related diseases such as diabetes, obesity, and neurodegenerative diseases is oxidative stress (50). Oxidative stress is defined by an imbalance of ROS and antioxidant activity that could lead to cell and tissue damage (49). Excess levels of mtROS, specifically superoxide, can lead to cell damage and mitochondrial-dependent apoptosis (57). Under oxidative stress, a pore is created in the inner mitochondrial membrane, halting OxPhos, leaking cytochrome C into the cytoplasm, and inducing apoptosis as a result of DNA fragmentation and ultimately ending in cell death (57, 58). However, not all ROS production is detrimental to cells, as low

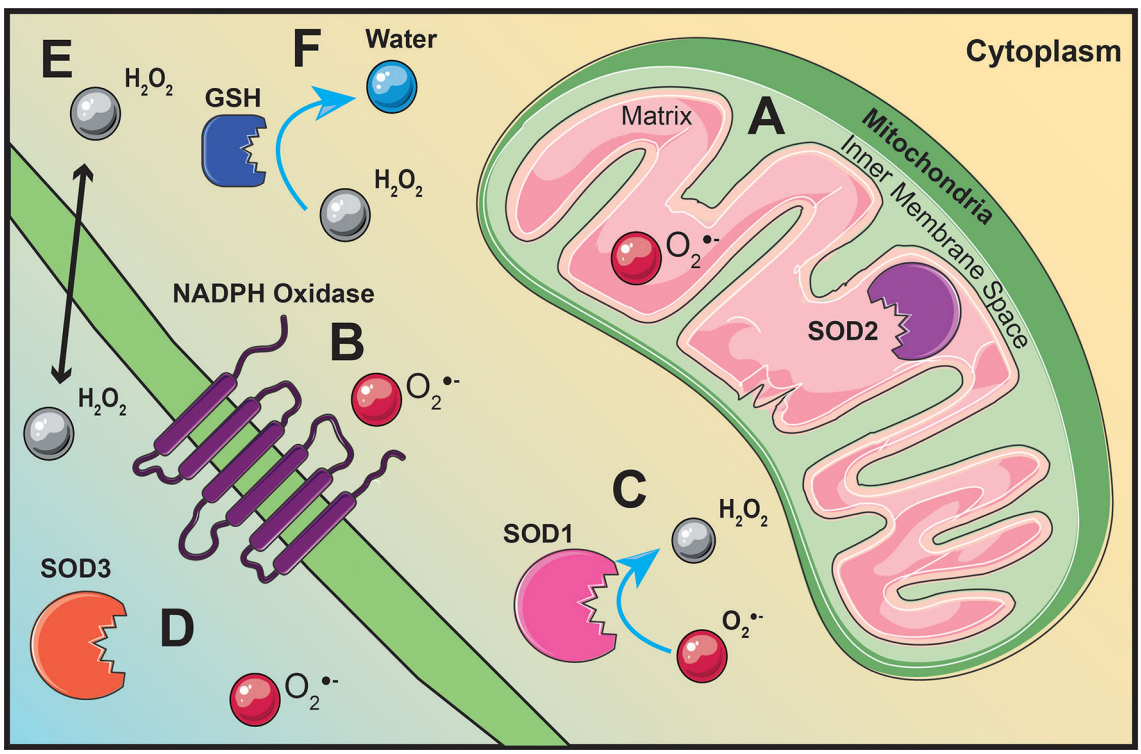


**FIGURE 3 | ROS production originates from various locations throughout the cell.** Superoxide is generated within the mitochondrial matrix **(A)** or by membrane bound NADPH oxidases **(B)**. Superoxide dismutase (SOD) located within the mitochondrial matrix **(A)**, within the cytoplasm **(C)**, or extracellularly **(D)** will convert superoxide into hydrogen peroxide ($H_2O_2$) **(C)**. Hydrogen peroxide is able to act as signaling molecule within the cell or extracellular due to its ability to diffuse across the cell membrane **(E)**. Glutathione (GSH), located throughout the cytoplasm, will regulate hydrogen peroxide levels by converting hydrogen peroxide into water **(F)**.

levels of hydrogen peroxide can activate pro-survival proteins such as Bcl-2 (57).

Mitochondrial dysfunction and the generation of oxidative stress could contribute to disease pathology in SLE, T1D, and RA (4, 50, 59–61). Increased antioxidant activity in patients with SLE improved mitochondrial metabolism, decreased mtROS, and reduced inflammation (59). In T1D, ROS synthesis can influence autoreactive T cell activation and directly mediate β-cell damage partly due to weak antioxidant defenses in β-cells (60). An inadequate antioxidant defense may contribute to oxidative stress within the β-cell, influencing its dysfunction and immunogenicity in T1D (62). Oxidative stress due to mitochondrial dysfunction could also enhance inflammatory responses in RA (61). Analysis of genes and protein-protein interactions in human patients with RA revealed that numerous mitochondrial-related pathways influence RA pathogenesis (61).

Mitochondrial DNA (mtDNA) deletions may manifest in a variety of syndromes, diseases, or phenotypes varying in severity (63, 64). mtDNA encodes for 13 proteins that are essential for OxPhos (64). In humans, it was revealed that the single nucleotide polymorphism (SNP) nt13708A in mtDNA significantly increased susceptibility to MS and SLE (65, 66). This SNP is in the *MT-ND5* gene, which encodes a subunit of complex I in the ETC (65, 66). While both studies identified nt13708A, the functional consequences of this SNP remain unknown. However, not all mtDNA SNPs result in increased disease susceptibility. In T1D, the C5173A SNP in the *mt-ND2* gene provided protection against disease development (67). Further characterization of *mt-Nd2*$^{a}$, an allelic variant of *mt-Nd2*, exhibited a reduction in ROS generated from complex I (67). These findings demonstrate that SNPs in mtDNA can modulate disease by altering OxPhos. Further studies on the effects of mtDNA deletions on OxPhos or superoxide generation could provide insight into the development of autoimmunity as a result of mitochondrial dysfunction.

Understanding the redox-dependent effects on immunometabolism and the generation of proinflammatory T cell responses needs to be further defined as a potential therapeutic strategy. The field of immunometabolism will define the molecular and cellular contributors that connect metabolism and immune cell function (14). Decades of research have shown nutrient availability and its effects on immune responses. Yet, only the last decade has provided insight on how metabolism coupled with ROS is a determinant of adaptive immune cell phenotype and effector responses.

## DIFFERENTIATION OF TH1/TH17 T CELLS IS PROMOTED BY GLYCOLYSIS AND STABILIZED BY ROS

Inflammatory responses prime Th1/Th17 differentiation by promoting a metabolic environment toward glycolysis. An inflammatory response often creates an environment depleted of nutrients and oxygen (68). Coupled with proinflammatory cytokines that will induce the activation of TF necessary for Th1/Th17 differentiation, the lack of oxygen results in increased glycolysis and skewed T cell differentiation (69–71). In addition to an enhanced glycolytic environment, ROS synthesis will stabilize RORγt TF expression to promote Th17 differentiation (72, 73). Increasing our understanding of the effects of glycolysis and ROS synthesis in Th1 and Th17 CD4 T cell differentiation could give rise to novel targets for delaying T cell-mediated autoimmune diseases.

Th1 induction begins with APCs secreting IL-12, a key cytokine in Th1 differentiation, to upregulate proinflammatory cytokine IFN-γ secretion and TF T-bet expression (74). Deficiency in IL-12 will reduce autoreactive Th1 responses, including IFN-γ production, as a result of disrupting a feedback loop in activated T cells (75). The induction of this feedback loop is key to Th1 differentiation since IFN-γ is a proinflammatory cytokine that will upregulate other cytokines necessary for Th1 differentiation including IL-12 (70, 71).

Th1 differentiation is primarily driven by an upregulation of glycolysis to support expression of T-bet and IFN-γ (69–71). Differentiating T cells engaged in aerobic glycolysis preferentially translate *Ifng* mRNA *versus* those using OxPhos (76). Pyruvate, generated by glycolysis, is utilized by lactate dehydrogenase A, which also supports aerobic glycolysis in activated T cells in order to promote proinflammatory IFN-γ production (69). This suggests that glycolytic upregulation will promote proinflammatory cytokines that shift naïve CD4 T cell activation toward Th1 differentiation (69–71). Therefore, it is not surprising that cytokine-producing Th1 cells are diminished when the inability to transport glucose for glycolysis is facilitated by a Glut-1 deficiency (77). In T1D, there is an abundance of glucose, which could be contributing to an environment that preferentially drives Th1 differentiation.

IFN-γ production by autoreactive Th1 cells is involved in the destruction of insulin-secreting β-cells in T1D (78) (**Figure 1**). During insulitis, APCs infiltrate islets and migrate to peripheral lymphoid tissues where both naïve autoreactive CD8 and CD4 T cells are activated (9). Following activation, T cells migrate back to the islets located within the pancreas (79). Once in the pancreas, activated Th1 cells produce IL-2 and IFN-γ to mature cytotoxic CD8 T cell responses (80). The presence of other cytokines, such as IL-1β, TNF-α, and IFN-γ, promote Th1 differentiation in addition to inhibiting insulin secretion and inducing β-cell apoptosis (81, 82). Although both CD8 and CD4 T cells contribute to β-cell destruction, a deficiency of CD4 T cells protects against spontaneous T1D development in NOD mice (83). Therefore, reducing glycolysis and/or ROS production in autoreactive Th1 CD4 T cells represents a potential therapeutic strategy for delaying T1D.

Insulin is a key hormone that is secreted from β-cells in response to high glucose, impacting metabolism by signaling cells to uptake glucose and undergo glycolysis (84). Insulin signaling requires insulin-induced hydrogen peroxide generation to maintain autophosphorylation of insulin-stimulated insulin receptor (73). Inhibition of superoxide generation by complex II of the ETC prevents hydrogen peroxide generation after insulin stimulation, disrupting insulin signaling and glucose uptake (85). Decreasing glycolytic signaling in T cells by regulating hydrogen

peroxide to decrease glucose uptake may be another immunotherapy used to orchestrate a metabolic blockage. The use of the glycolytic inhibitor 2-deoxy glucose (2-DG), has shown promise in diminishing autoreactive CD4 T cell responses without harming other aspects of the immune system (34). This would be beneficial compared to other potential treatment options, such as rapamycin, which acts as a universal immunosuppressant targeting APCs and T cells (86). Rapamycin is a mTOR inhibitor that would prevent all cells from being able to monitor for DNA damage, synthesize proteins, and coordinate an appropriate response to intracellular stress if administered systemically (87). This could alter immune function, lipid homeostasis, and muscle mass homeostasis resulting in increased susceptibility to other diseases (87). Therefore, there is a need for the development of targeted metabolic therapies specific for autoreactive T cells without impairing the ability of the immune system to mount defenses against microbial pathogens.

Th17 cells, like Th1 cells, also rely on glycolysis for their differentiation. Currently, there are studies showing the promise of this type of metabolic targeting therapy in Th17-mediated diseases including MS (88). Suppression of glycolysis *via* deletion of Glut1 has been shown to protect mice from Th1 and Th17 cell-driven diseases such as colitis and inflammatory bowel disease by decreasing the expansion and survival of T cells (77). A possible target for diminishing glycolysis in Th17 cytokine responses would be decreasing hypoxia-inducible factor (HIF)-1α expression. HIF-1α is a TF that will contribute to metabolic programming of activated T cells by mediating a switch from OxPhos to aerobic glycolysis (89, 90). HIF-1α will specifically stimulate Th17 differentiation by binding to the RORγt promoter to increase IL-17A production (89). Prostaglandin E2 ($PGE_2$) will boost glycolytic activity by stabilizing HIF-1α (91), while simultaneously skewing T cells away from a Th1 phenotype by inhibiting IFN-γ production (92). Prevention of hypoxic environments that will foster glycolytic activity *via* $PGE_2$ inhibition is a current treatment option for Th17-mediated autoimmune diseases including RA (92).

In MS, pathogenesis is driven by pathogenic Th17 cells interacting with the central nervous system (CNS) (93) (**Figure 1**). Naïve T cells are primed outside the CNS by APCs, cross the blood-brain barrier, and produce proinflammatory responses that result in the destruction of myelin and axons (93). Experimental autoimmune encephalomyelitis (EAE) is a mouse model used to study MS and studies have shown that increased ROS during pathogenic Th17 differentiation is another possible metabolic target for MS (94). Inflammatory Th17 responses are promoted by hydrogen peroxide stabilizing insulin receptor signaling to activate HIF-1α (72, 73). T cells deficient in insulin receptor signaling will have a diminished inflammatory response when activated, demonstrating the importance of insulin receptor stabilization by hydrogen peroxide for pathogenic Th17 differentiation (72, 95). Additionally, hydrogen peroxide contributes to Th17 differentiation by activating HIF target genes and stabilizing HIF-1α proteins (96). By inhibiting mitochondrial OxPhos during Th17 activation, mTOR signaling and the expression of basic leucin zipper transcriptional factor ATF-like (BATF) will be decreased (97). Without BATF to increase chromatin accessibility of Th17 transcription factors, mice displayed a resistance to EAE without affecting Th1/Th2 effector responses (98). This observation highlights the promise of metabolic therapies targeting ROS production in autoreactive Th17 cells, but immunometabolic reprogramming can also affect Treg differentiation as another potential avenue to delay EAE disease onset (97).

## OXPHOS IS REQUIRED FOR TREG/TH2 DIFFERENTIATION AND TREG SUPPRESSIVE FUNCTION

It has been debated whether the loss of Treg function and/or the number of Treg cells is a major contributor in T cell-mediated autoimmune diseases such as T1D, MS, and RA (99). Tregs have a broad set of functions including ensuring tolerance to autoantigens, limiting excess immune responses, and providing homeostasis of various tissues (100). Their development is similar to conventional T cells, but they are unique in their underlying self-reactivity during thymic selection, allowing them to suppress other CD4 T cell effector populations (2, 101). Strategies to increase the number of Tregs and enhance immunosuppressive Treg function are ideal approaches to restore peripheral tolerance in autoimmune diseases.

Therefore, studies to determine how immunometabolism contributes to Treg homeostasis, differentiation, and function are worthy research endeavors. Loss of the anti-apoptotic factor, Bcl-2, did not alter the quality or quantity of Tregs isolated from mice (102). However, increased expression of Bcl-2 by IL-7 enables natural Tregs to survive and proliferate properly while they circulate between secondary lymphoid organs (100). Bcl-2 is known to increase mtROS superoxide production in complexes I and III within the ETC (103). Tregs deficient in complex III-derived superoxide are able to maintain stable Foxp3 expression, cell proliferation, and survival, yet their suppressive capacity is lost (104, 105). This paralleled loss of function emphasizes the importance of proper mitochondrial metabolism throughout T cell activation and homeostasis.

There is a close developmental relationship between Treg and Th17 cells. TGF-β alone will induce Treg differentiation, however if IL-6 is present, Th17 cells will be preferentially induced (5). IL-6 will promote glucose uptake and metabolism (106, 107). This is due to IL-6 terminating Foxp3-mediated inhibition of Th17 TF RORγt (107). Foxp3 is a lineage-specific TF for Treg cells (3) (**Figure 1**). In the absence of other proinflammatory cytokines, Foxp3 inhibits Th17 differentiation by blocking RORγt activity in activated T cells (108). IL-2 negatively regulates IL-17 production while driving Treg proliferation, differentiation, and function (109, 110). In SLE, due to the reduction of IL-2, there is a decrease in the Treg population (111). In the absence of HIF-1α, a Th17 promoting

factor, Th17 cytokine responses will be dampened and Foxp3 Treg differentiation will be elicited instead (97). This could be due to the absence of the Foxp3 ubiquitin-mediated degradation pathway that is regulated by HIF-1α (112). HIF-1α increases RORγt expression while simultaneously tagging Foxp3 protein for ubiquitination (89).

Myelocytomatosis oncogene (Myc) is a TF that drives metabolic reprogramming toward glycolysis (34). Expression of Foxp3 suppresses glycolysis by binding to the Myc promotor and suppressing Myc gene expression (34). Diminishing glycolysis *via* Glut1deficiency does not affect Treg differentiation or cell numbers (77) since Treg cells utilize fatty acid oxidation (FAO) for Treg differentiation (113). By suppressing glycolysis, Foxp3 reactivates OxPhos, which is essential for Treg metabolism, stability, and function (**Figure 1**).

Similar to Tregs, Th2 cells upregulate fatty acid lipid metabolism and inhibition of these metabolic pathways will reduce a Th2-mediated response (114). The key role of OxPhos in Th2 differentiation is highlighted by a study that showed that reduced mtROS produced by complex-I inhibited anti-CD3-induced IL-2 and IL-4 expression and stunted Th2 differentiation (27). The production of IL-4 aids in the differentiation of naïve T cells toward the Th2 phenotype by inducing expression of the key TF Gata3 (8, 115). Expression of Gata3 is sufficient to induce the Th2 phenotype because it creates a positive feedback loop by inducing IL-4, which maintains Th2 cell identity (114). Increased activity of the kinase mTOR is present in all effector Th lineages in order to coordinate cell proliferation and metabolism (87). Unique to Th2 cells is the increase in fatty acid uptake by peroxisome proliferator activated receptor gamma (PPAR-γ) that also regulates fatty acid metabolism (114). Both IL-4 and mTOR promote PPAR-γ in Th2 cells and its absence will not only affect OxPhos, but Th2 function as well (114). Unfortunately, Th2 differentiation is diminished in individuals taking rapamycin due to its inhibitory effects on mTOR activity (87). Loss of Th2 function *via* decreased mTOR could be associated with decreased PPAR-γ binding to key genes such as *Gata3, Il5*, and *Stat5* (116).

Th2 cells protect from parasitic helminths infections and stimulate repair of damaged tissue repair (117). While there are no Th2-mediated autoimmune diseases, modulating the balance between Th1 and Th2 cells can affect autoimmune responses in animal models of RA (118). Treatment of arthritic mice with IL-4 suppressed disease, but did not reverse disease progression (118). Similar success was also observed in mouse models of T1D that skewed T cell responses toward a Th2 phenotype in an effort to prevent the activation of autoreactive Th1 cytokine responses (119). The efficacy of Th2 skewing has not been investigated in mouse models of MS or SLE. However, the loss of the nuclear factor erythroid 2-related factor 2 (NRF2) TF involved in inducing an antioxidant response, diminished Th2 cytokine responses and concomitantly, increased Th1 differentiation and SLE (120). While Th2 skewing may delay autoimmunity, Th2 cells also contribute to asthma and allergy. Allergen immunotherapy involves continuous exposure to allergens at increasing concentrations and is the only well-established treatment for asthma that targets Th2 cells (121). While the mechanism is still undefined, allergen immunotherapy has been shown to skew T cell differentiation away from a Th2 phenotype toward a Treg or Th1 phenotype (122).

## DECREASING PATHOGENIC CD8 T CELLS IN AUTOIMMUNITY BY PROMOTING OXPHOS

Similar to CD4 T cells, CD8 T cell activation requires antigen presentation, co-stimulatory molecule interactions, and the synthesis of cytokines and ROS (123, 124). However, unlike CD4 T cells, MHC-I is required for CD8 TCR stimulation, and deficiency in co-stimulatory molecules such as CD40L and CD28 does not result in anergy or decreased proliferation (125). Additionally, despite an increase in glycolysis to support clonal expansion, a deficiency in Glut1 expression does not affect CD8 T cell proliferation after activation (77, 126). Rather, their role in viral clearance is maximized by proinflammatory cytokines such as IFN-γ and IL-12, because it shifts activation toward short-lived effector cell (SLEC) populations (123).

The two main subsets of CD8 T cells are SLEC and memory precursor effector cells (MPEC) (123). Following TCR stimulation, mitochondrial fusion occurs in CD8 T cells to support increased OxPhos that promote cell proliferation (127, 128). Halting OxPhos *via* the deletion of AMP-activated protein kinase (AMPK) promotes the activation of the mTOR pathway and subsequently, defective CD8 T cell memory differentiation (129, 130). Inhibition of mTOR through AMPK activity improves the quality of CD8 memory T cells by accelerating effector to memory transition without affecting CD8 T cell effector function (129, 131). The effect of AMPK on CD8 T cell differentiation resulted in an interest in AMPK as a possible target for CD8 T cell memory generation. AMPK, in addition to inhibition of growth regulator mTOR, also regulates mitochondrial fission/fusion and promotes mitochondrial biogenesis (132). Due to memory T cells having an increased mitochondrial mass, AMPK regulation of mitochondrial biogenesis could contribute to increasing mitochondrial mass promoting memory T cells rather than cytotoxic CD8 T cells (133).

In T1D, APCs such as macrophages and dendritic cells are the first immune cells that infiltrate islets and contribute to pancreatic β-cell destruction (134). Macrophages activate Th1 CD4 T cells to produce IL-2 and IFN-γ to facilitate the differentiation of CD8 T cells to become cytotoxic (80). IL-1β secretion by APCs can function as a proinflammatory third signal for T cell maturation and enhance antigen-specific CD8 T cell effector function by increasing cytotoxic activity and IFN-γ production (135). If MHC-I is not expressed on β-cells, mice were protected from diabetes development (134). Therefore, strategies that can inhibit CD8 T cell effector responses would be beneficial as a T1D therapeutic. Recent investigations have tested the efficacy of 2-DG to inhibit glycolysis and autoreactive

CD8 T cell cytolytic responses in T1D (136). Inhibition of glycolysis decreased islet infiltration of pathogenic CD8 T cells (136), but the effects of 2-DG on cytotoxic CD8 T cell effector responses were not determined.

In MS, CD8 T cells are a viable target for therapy (137). Pathogenic CD8 T cells have been located in MS plaques, cerebrospinal fluid, and demyelinated axons (137). Mutations in MHC-I, along with gradual upregulation of MHC-I on various cell types throughout disease progression are associated with increased risk for MS and may also contribute to the increased ratio of CD8 to CD4 T cells in MS brain lesions (138). The accumulation of the glycolytic byproduct, lactate, in the cerebrospinal fluid of MS patients compared to controls has recently been considered an indicator of disease progression (139). Promoting a metabolic shift to decrease glycolytic activity may provide protection from disease progression. Lactic acid is able to promote OxPhos by inducing glycolysis/OxPhos interconversion to suppress cytotoxic CD8 T cell proliferation and cytokine production (140, 141). These biomarkers and different metabolic indicators for disease may all be therapeutic options worth experimentally perusing in order to decrease pathogenic CD8 T cell activity.

## DECREASED ANTIOXIDANT ACTIVITY IN T CELL-MEDIATED DISEASES CAN BE UTILIZED AS A BIOMARKER OR THERAPY FOR AUTOIMMUNITY

Antioxidants are effective in decreasing oxidative stress and loss of antioxidant activity may also be involved in autoimmune diseases. Glutathione (GSH) is a tripeptide antioxidant consisting of glutamate, cysteine, and glycine, located within the cytoplasm and utilized by the cell to detoxify ROS such as hydrogen peroxide (142, 143) (**Figure 3F**). The reductive capability of the sulfhydryl group contained within the cysteine makes GSH a pivotal component of protecting the cell from oxidative damage and stress resulting from excess ROS (143). Decreased activation of the NRF2 pathway, which regulates antioxidant genes including GSH, is observed across many T cell-mediated diseases (144). This is most likely due to GSH supporting activation-induced metabolic reprograming in T cells. By decreasing ROS, GSH reinforces glycolytic metabolic reprogramming that is required by inflammatory Th1 and Th17 cytokine responses (145). Studies have also shown that a deficiency in GSH results in impaired IFN-γ and IL-17 cytokine production (146).

Hyperglycemia is associated with increased oxidative stress, free radical production, suppression of antioxidant function, and is also a common complication in T1D and type 2 diabetes (T2D) (49, 147, 148). Furthermore, increased cytosolic hydrogen peroxide generation in pancreatic islets as a result of decreased GSH and SOD in individuals with T2D leads to impaired insulin secretion (149, 150). Children diagnosed with T1D show significantly decreased GSH antioxidant capacity compared to age-matched healthy children (147, 151). Therefore, it is encouraging that NRF2 activators have shown a great deal of promise as a therapeutic in pre-clinical trials for diabetic complications associated with increased oxidative stress (152).

GSH deficiency is also prevalent in MS (153). Compared to adjacent white matter, there is a significant reduction in GSH in MS plagues (153). GSH delivery to the CNS is difficult due to the inability to cross the blood-brain barrier, short half-life, and the requirement of high doses to achieve a therapeutic effect (154). While oral GSH did not impact disease progression, there are clinical trials to investigate NRF2 activation as a potential therapeutic in MS to increase downstream expression of antioxidants such as GSH (154). A phase 3 clinical trial for MS demonstrated that oral treatment with delayed-release dimethyl fumarate (DMF) was effective in activating NRF2, but further studies are needed to determine if GSH levels were increased and if MS could be delayed following DMF treatment (155).

In mouse models of RA, overexpression of SOD3 suppressed proinflammatory cytokine production and disease development (156). Unfortunately, human translation is limited due to a short half-life and instability of SOD (157). Interestingly, saliva taken from individuals with SLE displayed decreased presence of SOD compared to healthy controls (158). Therefore, it has been proposed that decreased SOD activity could alternatively be utilized as a biomarker for SLE (158). Other investigators have employed SOD mimetics in mouse models of T1D to increase stability and longevity of antioxidant activity in an effort to delay pancreatic β-cell destruction (159). The use of SOD mimetics can delay the adoptive transfer of T1D with a diabetogenic CD4 T cell clone that was partly due to a decrease in T cell proliferation and IFN-γ production (160). Similar results were also observed when CD8 T cells were treated with a SOD mimetic resulting in decreased proliferation, cytokine production and cytolytic effector responses (124). The ability of SOD mimetics to delay T1D progression and inhibit autoreactive T cell responses suggests these novel antioxidants may also be applicable for the treatment of SLE and RA in future studies.

## CONCLUSION

Increasing our understanding of the role of immunometabolism in T cell-mediated autoimmune diseases may provide novel insights into the redox-dependent mechanisms involved in immune dysregulation. Specifically, determining the homeostatic role of glycolysis and OxPhos in naïve and effector T cells may bridge the knowledge gap of metabolic reprogramming in T cell activation and differentiation. T1D, MS, and RA are autoimmune diseases that would benefit from metabolic reprogramming of autoreactive T cells to alleviate disease pathogenesis. Metabolically targeting glycolysis to decrease Th1 cytokine responses in T1D may prevent T cell-mediated β-cell destruction. Similarly, inhibiting glycolysis by targeting HIF-1α stabilizers such as hydrogen peroxide and $PGE_2$ can also dampen Th17 cytokine responses in MS to

delay neuronal damage. Decreasing glycolysis is not only effective in limiting autoreactive CD4 T cell responses, but can also diminish cytotoxic CD8 T cell effector responses in T1D and MS. Increasing our knowledge of the contribution of mtROS in T1D, MS, RA, and SLE and the effect of antioxidants to diminish mtROS may provide a novel immunotherapeutic approach to limit T cell-mediated autoimmunity in the near future.

## AUTHOR CONTRIBUTIONS

MC and HT conceived, wrote, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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# Targeting Tristetraprolin Expression or Functional Activity Regulates Inflammatory Response Induced by MSU Crystals

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The RNA-binding protein tristetraprolin (TTP) is an anti-inflammatory factor that prompts the mRNA decay of target mRNAs and is involved in inflammatory diseases such as rheumatoid arthritis (RA). TTP is regulated by phosphorylation, and protein phosphatase 2A (PP2A) can dephosphorylate TTP to activate its mRNA-degrading function. Some small molecules can enhance PP2A activation. Short interfering RNA (siRNA) targeting TTP expression or PP2A agonist (Arctigenin) was administered to monosodium urate (MSU) crystal-induced J774A.1 cells, and the expression of inflammatory related genes was detected by RT-PCR and Western blot assays. The effects of Arctigenin in mouse models of acute inflammation induced by MSU crystals, including peritonitis and arthritis, were evaluated. The data indicated that TTP expression levels and endogenous PP2A activity were increased in MSU-crystal treated J774A.1 cells. TTP knockdown exacerbated inflammation-related genes expression and NLRP3 inflammasome activation. However, PP2A agonist treatment (Arctigenin) suppressed MSU crystal-induced inflammation in J774A.1 cells. Arctigenin also relieved mitochondrial reactive oxygen species (mtROS) production and improved lysosomal membrane permeability in MSU crystal-treated J774A.1 cells. Moreover, TTP knockdown reversed the anti-inflammatory and antioxidant effects of Arctigenin. Oral administration of Arctigenin significantly alleviated foot pad swelling, the number of inflammatory cells in peritoneal lavage fluids and the production of IL-1β in the mouse model of inflammation induced by MSU crystals. Collectively, these data imply that targeting TTP expression or functional activity may provide a potential therapeutic strategy for inflammation caused by MSU crystals.

**Keywords: TTP, Arctigenin, NLRP3 inflammasome, mitochondrial ROS, autophagic flux 3**

## INTRODUCTION

Gout is caused by the deposition of MSU crystals in and around the joints (1). Currently, gout is the most common cause of inflammatory arthritis and its global epidemiology shows an increase in incidence and prevalence in both developed and developing countries (2). Acute gout episodes are clinically described as arthritic pain and inflammation that, if left untreated, can develop into recurrent acute urate deposition (gout) and progressive joint destruction affecting the patient's health (3, 4). However, to date, the clinical use of drugs has often resulted in undesirable side effects. Countless efforts have failed to create an effective and safe agent to treat gout.

Tristetraprolin (TTP, encoded by Ttp, also known as Zfp36) is one of the most characterized RNA binding proteins (RNA-BPs), and it mediates the instability of mRNA, recognizes ARE sequences through adjacent AUUUA-binding sites, and posttranscriptionally regulates the expression of tumor and inflammation-related genes (5). In the past decade, a large number of studies have shown that TTP plays an important role in balancing the inflammatory response (6) and plays a potential antitumor role by mediating the levels of proinflammatory cytokines such as IL-1β, TNF-α, IL-6 and COX-2 (7–9).

Some studies have revealed that TTP is regulated by p38 mitogen-activated protein kinase (MAPK). However, inhibiting the latter does not increase the level of unphosphorylated TTP (10, 11). Multiple inflammatory signaling pathways are regulated by PP2A, PP2A acts upstream of TTP signaling *in vitro* and *in vivo* (11, 12). PP2A activates TTP *via* dephosphorylation at S52 and S178, leading to the destabilization of target mRNAs (13). As agonist of PP2A, Arctigenin has anti-inflammatory effects (11, 12, 14). However, it is not clear whether TTP is involved in the inflammatory response induced by MSU crystals and whether PP2A activator can activate TTP and inhibit the inflammatory response induced by MSU crystals.

In this study, we found that TTP knockdown could aggravate the expression of inflammation-related genes induced by MSU crystals. However, Arctigenin, as a natural agonist of PP2A, can mitigate the inflammation induced by MSU crystals. The mechanisms may be involved in regulating mitochondrial function and oxidative stress as well as promoting autophagy flux, thereby inhibiting the activity of NLRP3 inflammasome in a TTP-dependent manner. More importantly, Arctigenin could protect against inflammatory models of gout in mice, such as peritonitis and arthritis.

**Abbreviations:** TTP, Tristetraprolin; MSU, monosodium urate; ATG, Arctigenin; RA, rheumatoid arthritis; PP2A, Protein phosphatase 2A; ROS, reactive oxygen species; SOD, superoxide dismutase; CAT, catalase; GPX, glutathione peroxidase; MtROS, mitochondrial reactive oxygen species; MMP, mitochondrial membrane potential; MPO, myeloperoxidase; HC, healthy control; AGA, acute gouty arthritis; IGA, intercritical gouty arthritis; Ctsb, cathepsin B; Ctsd, cathepsin D; iNOS, induce nitric oxide synthetase; NLRP3, NOD-like receptor family, pyrin domain containing 3; TNF-α, tumor necrosis factor α; TFEB, transcription factor EB; H&E, hematoxylin and eosin; IL-1β, interleukin 1 beta; PBMCs, Peripheral blood mononuclear cells; DHE, Dihydroethidium.

## MATERIALS AND METHODS

### Preparation of MSU Suspension

MSU crystals were prepared as previously described (15). One gram of uric acid (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 200 mL boiling water containing 1N NaOH, NaOH solution was added to adjust the pH to 8.9, and the solution crystallized overnight at room temperature. The precipitate was filtered out of the solution and dried at 42°C. The crystals were weighed under sterile conditions and suspended in PBS solution at a concentration of 25 mg/mL.

### Cell Culture and Transfection of siRNA

J774A.1 cells were cultured with DMEM containing 10% FBS. After the J774A.1 cells were transfected with scramble RNA (scRNA) or siRNA against the mouse TTP gene (siTTP) for 48 h, the cells were primed with LPS (100 ng/ml) for 1 h and subsequently challenged with the MSU suspension. Transfection of siRNA was performed using the INTERFERin siRNA Transfection Reagent following the manufacturer's instructions. The siRNA sequences were synthesized by Sango Biotech (Shanghai, China). The target mouse TTP siRNA sequences were as follows: 5'-CGACAAAGCAUCAGCU UCUTT-3' (sense) and 5'-AGAAGCUGAUGCUUUGUCGTT-3' (antisense).

### ELISA

IL-1β/IL-6/TNF-a levels were detected by an ELISA kit according to the manufacturer's instructions (NeoBioScience kit, Shenzhen, China).

### PP2A Activity Assay

PP2A activity was detected using the PP2A Immunoprecipitation Phosphatase Assay Kit (Merck Millipore, Darmstadt, Germany) following the manufacturer's instructions.

### Extraction of Protein From Cell Culture Supernatants for Detecting Caspase-1 and IL-1β by Immunoblotting

Cell culture supernatants were collected and precipitated in deoxycholate containing 20% trichloroacetic acid (20% TCA) and washed three times with 100% acetone, before concentration in 1× Laemmli buffer.

### Western Blot Analysis

RIPA buffer was applied to extract total protein from J774A.1 cells or foot pad tissue. Nuclear protein was extracted using the CellLytic™ NuCLEAR™ Extraction Kit (Sigma, USA). The protein concentration was detected using a BCA protein assay kit (Thermo Scientific, MA, USA). The proteins were suspended in 1×Laemmli Buffer, boiled at 95°C for 6 minutes and separated by SDS-PAGE. The proteins were transferred to a PVDF membrane. The membrane was incubated with the primary antibodies at 4°C overnight. After washing the membrane, the membrane was incubated with HRP-conjugated secondary antibody (BA1054, dilution 1:7500, BosterBio, Wuhan, China) for 1 h at room temperature. Finally the membrane was exposed to the gel

imaging system with a chemiluminescence kit and imaged with a gel imaging system. The density of protein bands was measured by ImageJ software. The ratio of target protein density to corresponding reference protein density is the relative expression of target gene in each group. The fold change of the treatment group was then calculated. Finally, statistical analysis was carried out. The details of the antibodies are in the **Supplementary File**.

## RNA Extraction and Quantitative Real-Time PCR

Total RNA was isolated from J774A.1 cells or foot pad tissue with TRIzol Reagent (TIANGEN, China), and cDNA was synthesized with a reverse transcriptase kit. A real-time system (Roche, USA) was used for PCR with SYBR Green Master Mix. The relative amounts of the target mRNA were normalized to the expression level of the reference gene GAPDH, and the data were analyzed through the $2^{-\Delta\Delta Ct}$ method. The primer sequences are shown in **Table 1**.

## Detection of Total and Mitochondrial Reactive Oxygen Species

Mitochondrial superoxide indicator (Mito-SOX, Invitrogen; Thermo Fisher Scientific, Inc., USA) and Mito-Tracker Green (40742ES50) co-localization was used to detect mitochondrial ROS by Laser confocal microscopy imaging analysis following the described protocol (16). Cellular and mitochondrial ROS were respectively measured using the fluorescence probe DHE (BB-47-51, BestBio, China) and Mito-SOX fluorescence probe (Invitrogen; Thermo Fisher Scientific, Inc., USA) were detected by FACS following the manufacturer's instructions. The mean fluorescence intensity (MFI) of PE-Texas Red-H was quantified using the FlowJo software.

## Mitochondrial Membrane Potential Detection

Mitochondrial membrane potential (MMP) was evaluated with JC-1 probe (Beyotime, Shanghai, China, C2006) following the manufacturer's instructions. Briefly, J774A.1 cells were stained with JC-1 probe using the Olympus Laser confocal microscope imaging analysis or FACS measurement. In the FACS analysis, the ratio of PE-H (MFI)/FITC-H (MFI) was used to indicate MMP alternation through FlowJo software.

## LysoTracker and Acridine Orange Staining

Lysosome staining was respectively performed with acridine orange (Solarbio, Life Science) and Lysosome LysoTracker™ Deep Red (ThermoFisher Scientific, L12492) following the manufacturer's instructions, and then capture the image and analyze fluorescence intensity using Laser confocal microscope.

## Determination of Intracellular Antioxidant Enzyme Activity

J774A.1 cells were washed with cold PBS for three times, and lysis buffer (20 mM Tris buffer pH 7.5, 150 mM NaCl and 1% Triton X-100) at 4°C for 15 min. The cell lysates were centrifuged, and the supernatants were collected. The activities of SOD, GSH-PX, and CAT were tested using the corresponding assay kits (Beyotime, China) according to the manufacturer's instructions. The protein content was measured by a BCA assay kit (Thermo Fisher Scientific) following the manufacturer's protocol, and the data are expressed as U/mg protein.

## Mice

Male C57BL/6 mice aged 6 to 8 weeks were purchased from the Dossy Experimental Animals Company (Chengdu, China). All animal experiments were conducted in accordance with the Guidelines for the Care and Use of Experimental Animals and were approved by the Ethics Committee of North Sichuan Medical College.

## MSU Crystals Induced Peritonitis and Arthritis Mice Model

To induce peritonitis, 1 h after oral ATG (Cayman Chemicals, #14913) administration 40 mg/kg; ATG dissolved in 5% DMSO,

**TABLE 1** | The primers used for quantitative PCR.

| Gene | Forward sequence (5′–3′) | Reverse sequence (5′–3′) |
|---|---|---|
| Mouse TTP | TCTCTGCCATCTACGAGAGCC | TCCTCCGAGGGATTCGGTTC |
| Mouse IL-1β | GAAATGCCACCTTTTGACAGTG | TGGATGCTCTCATCAGGACAG |
| Mouse IL-6 | CTGCAAGAGACTTCCATCCAG | AGTGGTATAGACAGGTCTGTTGG |
| Mouse TNF-α | CCTGTAGCCCACGTCGTAG | GGGAGTAGACAAGGTACAACCC |
| Mouse COX-2 | TGCACTATGGTTACAAAAGCTGG | TCAGGAAGCTCCTTATTTCCCTT |
| Mouse iNOS | GGAGTGACGGCAAACATGACT | TCGATGCACAACTGGGTGAAC |
| Mouse NLRP3 | ATTACCCGCCCGAGAAAGG | CATGAGTGTGGCTAGATCCAAG |
| Mouse TTP | CCGAATCCCTCGGAGGACTT | GAGCCAAAGGTGCAAAACCA |
| Mouse Lamp1 | CAGCACTCTTTGAGGTGAAAAAC | CCATTCGCAGTCTCGTAGGTG |
| Mouse Lamp2 | TGTATTTGGCTAATGGCTCAGC | TATGGGCACAAGGAAGTTGTC |
| Mice Ctsb | CAGGCTGGACGCAACTTCTAC | TCACCGAACGCAACCCTTC |
| Mouse Ctsd | GCTTCCGGTCTTTGACAACCT | CACCAAGCATTAGTTCTCCTCC |
| Mouse SOD1 | AACCAGTTGTGTTGTCAGGAC | CCACCATGTTTCTTAGAGTGAGG |
| Mouse SOD2 | CAGACCTGCCTTACGACTATGG | CTCGGTGGCGTTGAGATTGTT |
| Mouse GPX1 | CCACC GTGTA TGCCT TCTCC | AGAGAGACGCGACATTCTCAAT |
| Mouse CAT | TGGCACACTTTGACAGAGAGC | CCTTTGCCTTGGAGTATCTGG |
| Mouse GAPDH | AGGTCGGTGTGAACGGATTTG | GGGGTCGTTGATGGCAACA |

vehicle-treated mice were administrated orally 5% DMSO), mice were intraperitoneally injected with the MSU suspension (3 mg in 200 ml sterile PBS). Six hours after injection of MSU suspension, the mice were euthanized and peritoneal cavities were washed with 10 ml PBS. Peritoneal lavage fluids were analyzed for IL-1β production by ELISA and for lymphocyte recruitment by FACS using the leukocyte common antigen FITC-CD45, the macrophage marker PE-F4/80, the neutrophil marker APC-(Ly-6G and Ly-6C), basophilic granulocyte marker APC-CD63. FITC-CD45 (553079), PE-F4/80 (565410) and APC-(Ly-6G and Ly-6C) (553129) were obtained from BD BioScience. APC-CD63 (143906) was purchased from Biolegend.

The arthritis mouse model was given ATG orally. 1 h later, 1 mg of MSU (in 40 µl of PBS) was injected into the right foot pad and the same volume of PBS was injected into the left foot pad as the control. 24 h after injection of MSU suspension, the swelling of the foot pad was measured, and then the mice were sacrificed. Joint index evaluation was based on a method described previously (15). Part of the foot pad tissue was added with RIPA buffer to extract protein, and the rest of the foot pad tissue was fixed and sliced for hematoxylin and eosin (H&E) staining and immunofluorescence detection. The details of the antibodies are in the **Supplementary File**.

## Statistical Analysis

GraphPad Prism 8 software was used for statistical analysis. The data were expressed as mean ± SEM. Statistical analysis was performed using One-way ANOVA analysis of variance. $P < 0.05$ was regarded as significantly different by using LSD or Dunnett's T3 test.

# RESULTS

## MSU Crystals Stimulated the Expression of Tristetraprolin *In Vitro* and *In Vivo*

The murine macrophages cell line J774A.1 has been widely used as a cell model to study the activation of NLRP3 inflammasomes due to its expression of NLRP3, Caspase-1 and ASC (17, 18). To determine the influence of MSU crystals on TTP expression, J774A.1 cells were exposed to different doses of MSU crystals (25, 50 and 100 µg/ml) for 12 h, and the mRNA and protein levels of TTP were assessed. The quantitative RT-PCR and Western blot analysis data showed that MSU crystals increased TTP mRNA and protein expression in an almost dose-dependent manner (**Figures 1A, B**). Next, we sought to do a time course to assess the kinetics of MSU crystal-mediated TTP mRNA and protein levels

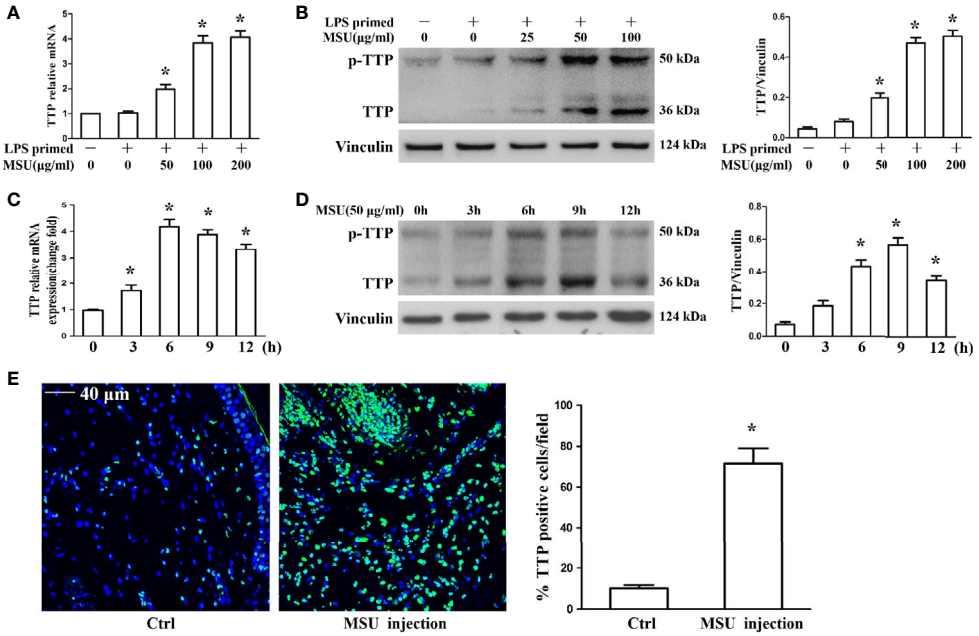


**FIGURE 1** | MSU crystals upregulated the expression of TTP *in vitro* and *in vivo*. **(A, B)** J774A.1 cells were primed with 100 ng/ml LPS for 1 h and then treated with different concentrations of MSU crystals for 12 h. **(A)** Total RNA was extracted from J774A.1 cells and reverse-transcribed, and the mRNA level of TTP was measured by qRT-PCR. *P < 0.05 *vs.* without MSU crystals treatment. **(B)** Total protein was isolated from J774A.1 cells, and TTP protein level was detected by Western blot. *P < 0.05 *vs.* without MSU crystals treatment. **(C, D)** J774A.1 cells were primed with 100 ng/ml LPS and then treated with 50 µg/ml MSU crystals at various time points (0, 3, 6, 9, 12 h). **(C)** Total RNA was extracted from J774A.1 cells, reverse-transcribed, and the mRNA level of TTP was measured by qRT-PCR. *P < 0.05 *vs.* without MSU crystals treatment. **(D)** Total protein was isolated from J774A.1 cells, TTP protein level was detected by Western blot. *P < 0.05 *vs.* without MSU crystals treatment. **(E)** Immunofluorescence assay was used to detect TTP protein distribution in mouse foot pad tissue sections. Blue shows nuclei stained with Hoechst 33342. Scale bar: 40 µm. (n=4 per group, mean ± SEM), *P < 0.05 *vs.* without MSU crystals injection in the foot pad tissue. All the data are expressed as the means ± SEM from n=3 independent experiments.

over time. Cells were exposed to 50 μg/ml MSU crystals for the indicated time periods (0, 3, 6, 9 and 12 h), and after cell lysis, RNA and protein were extracted for quantitative RT-PCR and Western blot detection, respectively. MSU crystals significantly enhanced TTP mRNA expression with a peak at 6 h (**Figure 1C**), but TTP protein expression reached its peak when the J774A.1 cells were stimulated with 50 μg/ml MSU crystals for 9 h (**Figure 1D**). Based on the established mouse model of gouty arthritis, the immunofluorescence data of mouse foot pad tissue sections showed that the number of TTP positive cells was significantly increased in the MSU crystal-treated group (**Figure 1E**), suggesting that TTP might be involved in MSU crystal-induced gouty arthritis.

## Blocking Tristetraprolin Expression Accelerates the MSU Crystal-Induced Inflammatory Response in J774A.1 Cells

We have found that MSU crystals can stimulate the expression of TTP. We further sought to demonstrate the effect of TTP knockdown on the expression of MSU crystal-induced inflammation-related gene. J774A.1 cells were transfected with TTP-targeted siRNA (siTTP) or scramble RNA (scRNA) and then stimulated with MSU crystals. As shown in **Figure 2A**, siRNA against TTP significantly alleviated MSU crystal-induced TTP mRNA expression. MSU crystal-induced TTP protein levels were similarly inhibited by siRNA against TTP, as detected by Western blot (**Figure 2B**). Transient primed LPS had little effect on the expression of IL-1β at the mRNA level (**Supplementary Figure 1**). Importantly, when TTP expression was inhibited, there was a corresponding increase in the mRNA expression of IL-1β, TNF-α, IL-6, COX-2 and iNOS in the MSU crystal-treated J774A.1 cells (**Figure 2C**). ELISA data also showed that after MSU crystals stimulation, the release of IL-1β, TNF-α and IL-6 in the culture supernatants from J774A.1 cells was upregulated, while these cytokines were further increased in MSU crystal-stimulated J774A.1 cells when TTP expression was inhibited (**Supplementary Figure 2**). In line with the RT-PCR data, Western blot analysis also revealed that the protein levels of COX-2 and iNOS in J774A.1 cells were enhanced after exposure to MSU crystals, and TTP knockdown further increased the iNOS and COX-2 protein levels in the MSU crystals stimulated J774A.1 cells (**Figure 2D**).

It has been reported that TTP can inhibit NLRP3 expression and NLRP3 inflammasome activation in LPS induced inflammation (19). The mRNA level of NLRP3 induced by MSU crystals remained almost unchanged by TTP knockdown (**Figure 2E**), but the MSU crystal treatment led to increased protein expression of NLRP3 (**Figure 2F**), suggesting that TTP mainly affected the translation of NLRP3 in the MSU crystals treated J774A.1 cells. Because TTP knockdown affected NLRP3 protein levels, we next determined whether it influenced the activation of the NLRP3 inflammasome. As expected, activation of the NLRP3 inflammasome in MSU crystal-induced J774A.1 cells resulted in the cleavage of Caspase-1 and IL-1β, and their release into the culture supernatant. Importantly, in TTP-specific siRNA transfected and MSU crystal-treated J774A.1 cells, the protein levels of both Caspase-1 and IL-1β secreted into the supernatants were elevated (**Figure 2G**).

## PP2A Agonist Attenuated MSU Crystal-Induced Inflammation in J774A.1 Cells

Because PP2A phosphatase activity is closely related to the anti-inflammatory function of TTP, we sought to investigate the effect of MSU crystals on PP2A phosphatase activity in J774A.1 cells. As shown in **Figure 3A**, when J774A.1 cells were treated with MSU crystals, PP2A phosphatase activity significantly increased, implying that PP2A activity may be related to inflammation induced by MSU crystals. Recently, it has been confirmed that Arctigenin can increase PP2A activation (14). We sought to determine the effect of Arctigenin on PP2A activity in J774A.1 cells stimulated by MSU crystals. Arctigenin also greatly augmented PP2A activity induced by MSU crystals (**Figure 3A**). We further studied the effect of Arctigenin on the MSU crystal-induced expression of inflammation-related genes. Arctigenin relieved the mRNA expression of inflammation-related genes induced by MSU crystals (**Figure 3B**). Consistent with the RT-PCR data, treatment with Arctigenin suppressed the secretion of IL-1β, TNF-α and IL-6 in culture supernatants from in J774A.1 cells (**Supplementary Figure 3**).Western blot data revealed that Arctigenin treatment significantly reduced the protein levels of COX-2 and iNOS induced by MSU crystals in J774A.1 cells (**Figure 3C**). Meanwhile, the production of Caspase-1 and IL-1β in culture supernatants was also suppressed (**Figure 3D**), suggesting that suppressed the activity of the NLRP3 inflammasome *in vitro*. All these data imply that Arctigenin can inhibit the inflammatory response induced by MSU crystals *in vitro*.

## Tristetraprolin Knockdown Abrogates the Arctigenin-Mediated Inhibition of the MSU Crystal-Induced Inflammatory Response

Thus far, we have found that TTP can regulate inflammation induced by MSU crystals, and Arctigenin can regulate PP2A activity and attenuate MSU crystal induced inflammatory gene expression *in vitro*. We hypothesized that Arctigenin might regulate its anti-inflammatory effects in a TTP-dependent manner. To confirm this link, we investigated the effect of Arctigenin on TTP expression. As shown in **Figure 4A**, MSU crystals significantly augmented TTP mRNA expression; however, this effect was unaffected by pretreatment with Arctigenin. We then examined the effect of Arctigenin on TTP protein levels. Because TTP protein has anti-inflammatory function when it is dephosphorylated, we speculate that Arctigenin may promote the level of dephosphorylated TTP protein. This was exactly what was observed in **Figure 4B**. Previous study indicated that phosphorylation of ERK and MAPK p38 in macrophages was significantly increased after MSU crystal stimulation (20). MAPK p38, when activated, in turn activates the downstream kinase MAPK activated protein kinase 2 (MK2). MK2 phosphorylates 52 and 178 serine of mouse TTP. ATG treatment did not affect extracellular signal-regulated kinase (ERK1), MAPK p38 or MK2 phosphorylation in

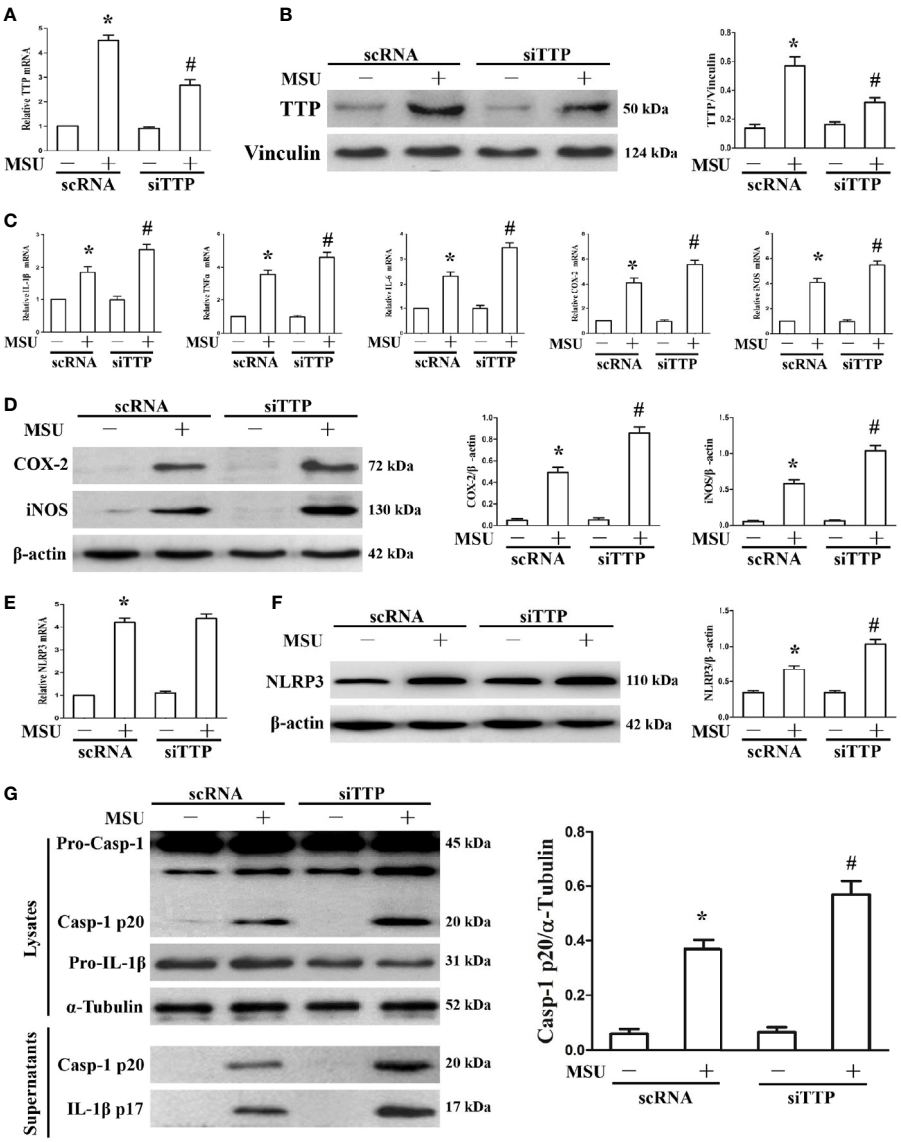


**FIGURE 2** | Influence of blocking TTP expression on the MSU crystal-induced inflammatory response in J774A.1 cells. **(A, B)** J774A.1 cells were transfected with scramble RNA (scRNA) or TTP-targeted siRNA (siTTP) for 48 h, primed with 100 ng/ml LPS and then stimulated with MSU crystals (50μg/ml) for 9 h. The knockdown of TTP mRNA and protein expression was confirmed by **(A)** RT-PCR and **(B)** Western blot (compared to α-tubulin as a loading control). **(C)** IL-1β, TNF-α, IL-6, COX-2 and iNOS mRNA expression was measured. **(D)** Western blot analysis of COX-2 and iNOS protein levels. **(E)** Effect of TTP knockdown on the NLRP3 mRNA level. **(F)** Impact of TTP knockdown on the NLRP3 protein level. **(G)** Effect of TTP knockdown on the protein levels of the p20 subunit of Caspase-1 and IL-1β in the cell lysates and culture supernatants. All the data are expressed as the means ± SEM from n=3 independent experiments, *P < 0.05 *vs.* without MSU crystals treatment; #P < 0.05 *vs.* scRNA transfection + MSU crystals treatment.

response to MSU crystals (**Figure 4C**). Therefore, we speculated that the anti-inflammatory effects of ATG *in vitro* might depend on its ability to modulate the TTP phosphorylation state but do not involve the impairment of signaling events upstream of TTP phosphorylation. To confirm the relationship between PP2A activation and TTP-mediated expression of inflammation-related gene, we explored whether Arctigenin-mediated inhibition depended on TTP by knocking down TTP and observing the effect on the expression of inflammation-related gene. TTP knockdown reversed the ATG-mediated inhibition of NLRP3 inflammasome activity (**Figure 4D**) and inflammation-related genes at mRNA expression levels (**Figure 4E**). Consistent with the RT-PCR data, the ELISA data also indicated that specific knockdown of TTP with siRNA abrogated the suppression of IL-1β, TNF-α and IL-6 protein secretion by Arctigenin (**Supplementary Figure 4**). These data further support our hypothesis that PP2A activators may mediate their anti-inflammatory effects in a TTP-dependent manner.

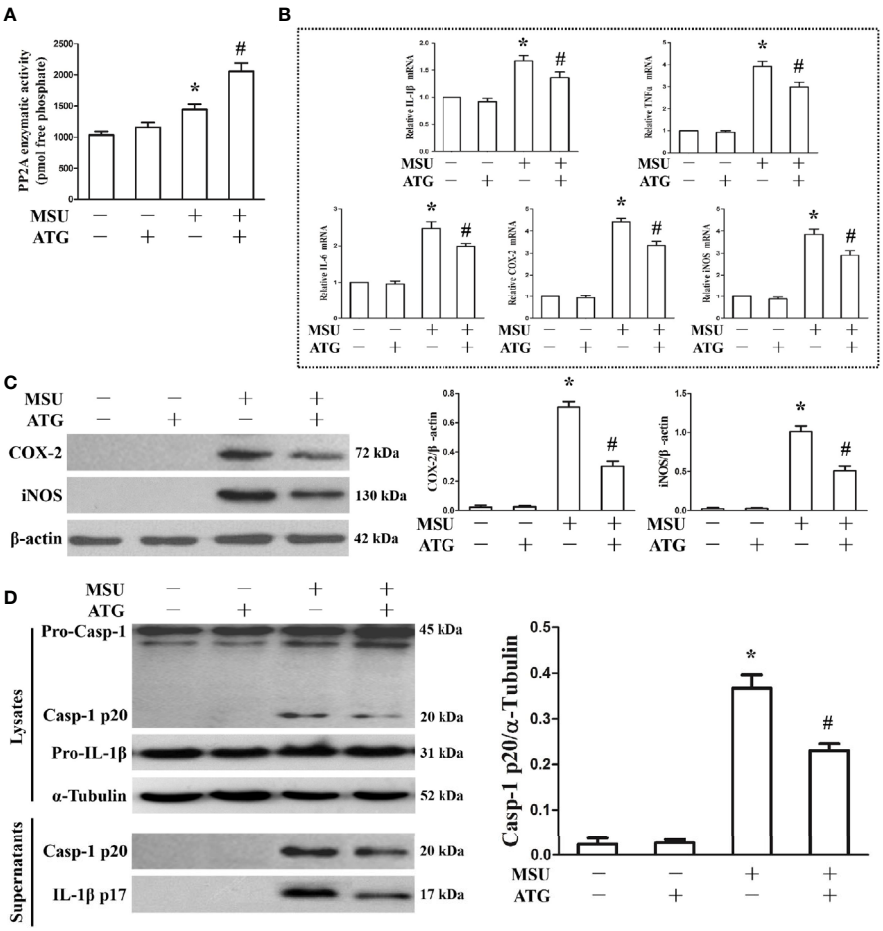


**FIGURE 3** | Effects of PP2A agonist (ATG) on PP2A phosphate activity and inflammation-related gene expression in MSU crystals stimulated J774A.1 cells. **(A–D)** J774A.1 cells were pretreated with ATG (5 μM) for 1 h, primed with LPS (100 ng/ml) for 1 h and then treated with MSU crystals (50 μg/ml) for 9 h. **(A)** Influence of a PP2A agonist (ATG) on MSU crystal-induced PP2A phosphatase activity. **(B)** The mRNA levels of IL-1β, TNF-α, IL-6, COX-2 and iNOS were detected using RT-PCR because of ATG treatment. **(C)** Western blotting was used to detect COX-2 and iNOS protein levels after ATG treatment. **(D)** The protein levels of the p20 subunit of Caspase-1 and IL-1β in the cell lysates and culture supernatants were measured using Western blot. All the data are expressed as the means ± SEM from n=3 independent experiments, *P < 0.05 *vs.* without MSU crystals treatment; #P < 0.05 *vs.* MSU crystals treatment + vehicle.

## Arctigenin Ameliorates Mitochondrial Dysfunction Induced by MSU Crystals

The involvement of ROS in the pathological process of acute gout has gained increasing recognition; moreover, it has been reported that Arctigenin has antioxidant functions (21). Hence, we explored the effect of Arctigenin on MSU crystal-induced oxidative stress. As shown in **Figures 5A, B**, we found that intracellular total ROS generation in the MSU crystal-treated J774A.1 cells increased up to almost 3-fold compared to that in the control cells. However, Arctigenin treatment dramatically attenuated the effect of MSU crystals on ROS generation. TTP knockdown reversed the regulation of ATG on the intracellular total ROS generation (**Figure 5C**). Intracellular ROS are mainly derived from mitochondria, so we further investigated the effect of ATG on mitochondrial ROS through mitochondrial superoxide indicators. The increase in mitochondrial ROS triggered by MSU crystals was blocked by Arctigenin treatment (**Figures 5D, E**). The regulation of ATG on mitochondria ROS was affected in TTP knockdown macrophages (**Figure 5F**). It has been reported that elevated mitochondrial ROS can damage mitochondria. To evaluate the level of mitochondrial damage, the mitochondrial membrane potential (ΔΨm) was detected using the fluorescent probe JC-1. MSU crystals significantly depolarized the mitochondrial membrane in J774A.1 cells, as shown by the shift in JC-1 fluorescence from red to green. Intervention with Arctigenin greatly relieved the damage of mitochondrial membrane potential caused by MSU crystals (**Figures 6A, B**). However, the improvement of mitochondrial membrane potential by ATG was impeded in TTP knockdown macrophages (**Figure 6C**). These data suggest that ATG can ameliorate the mitochondrial dysfunction induced by MSU crystals in a TTP-dependent manner.

To further elucidate the antioxidant effects of Arctigenin in MSU crystal-stimulated J774A.1 cells, we investigated the effects

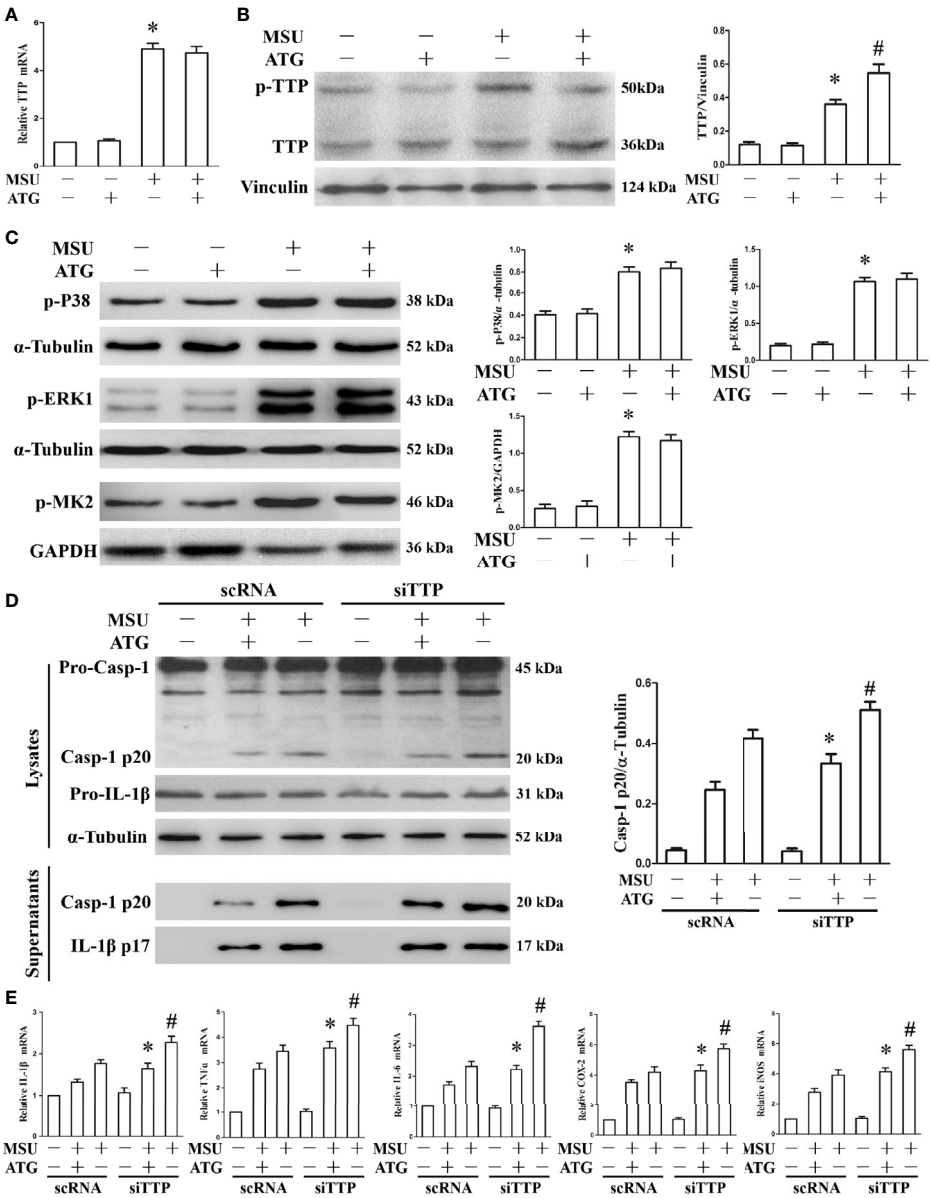


**FIGURE 4** | The effects of ATG on TTP expression and TTP knockdown abolishes the ATG-mediated repression of the MSU crystal-induced inflammatory response. **(A–C)** J774A.1 cells were pretreated with ATG (5 μM) for 1 h, primed with LPS for 1 h and then treated with MSU crystals (50 μg/ml) for 9 h. Total RNA and protein in the cells were extracted. *P < 0.05 vs. without MSU crystals treatment; #P < 0.05 *vs.* MSU crystals treatment + vehicle. **(A)** The mRNA expression level of TTP was detected using RT-PCR. **(B)** Western blotting was used to measure the protein level of TTP. **(C)** The phosphorylation levels of MAPK p38, ERK1 and MK2 were tested using Western blot assays. **(D, E)** J774A.1 cells were either transfected with scRNA or siTTP for 48 h, treated with 5 μM ATG for 1 h, primed with LPS for 1 h, and then stimulated with MSU crystals (50 μg/ml) for 9 h. **(D)** The protein levels of the p20 subunit of Caspase-1 and IL-1β in the cell lysates and culture supernatants were measured using Western blot. All the data are expressed as the means ± SEM from n=3 independent experiments, *P < 0.05 *vs.* MSU crystals treatment + ATG + scRNA; #P < 0.05 *vs.* MSU crystals treatment + scRNA. **(E)** The mRNA levels of IL-1β, IL-6, TNF-α, COX-2 and iNOS were determined. *P < 0.05 *vs.* MSU crystals treatment + ATG + scRNA; #P < 0.05 *vs.* MSU crystals treatment + scRNA. All the data are expressed as the means ± SEM from n=3 independent experiments.

of Arctigenin on the activity and expression level of intracellular antioxidant enzymes. The activities of SOD, GPX and CAT in MSU crystal-treated cells were significantly lower than those in the control (**Supplementary Figure 5A**). However, Arctigenin treatment significantly relieved the MSU crystal-induced decrease in antioxidant enzymes activities (**Supplementary Figure 5B**). The mRNA expression levels of antioxidant defense-related genes, including SOD1, SOD2, GPX1 and CAT were also detected. A significant decrease in the mRNA expression of SOD1, SOD2, GPX1 and CAT was observed in

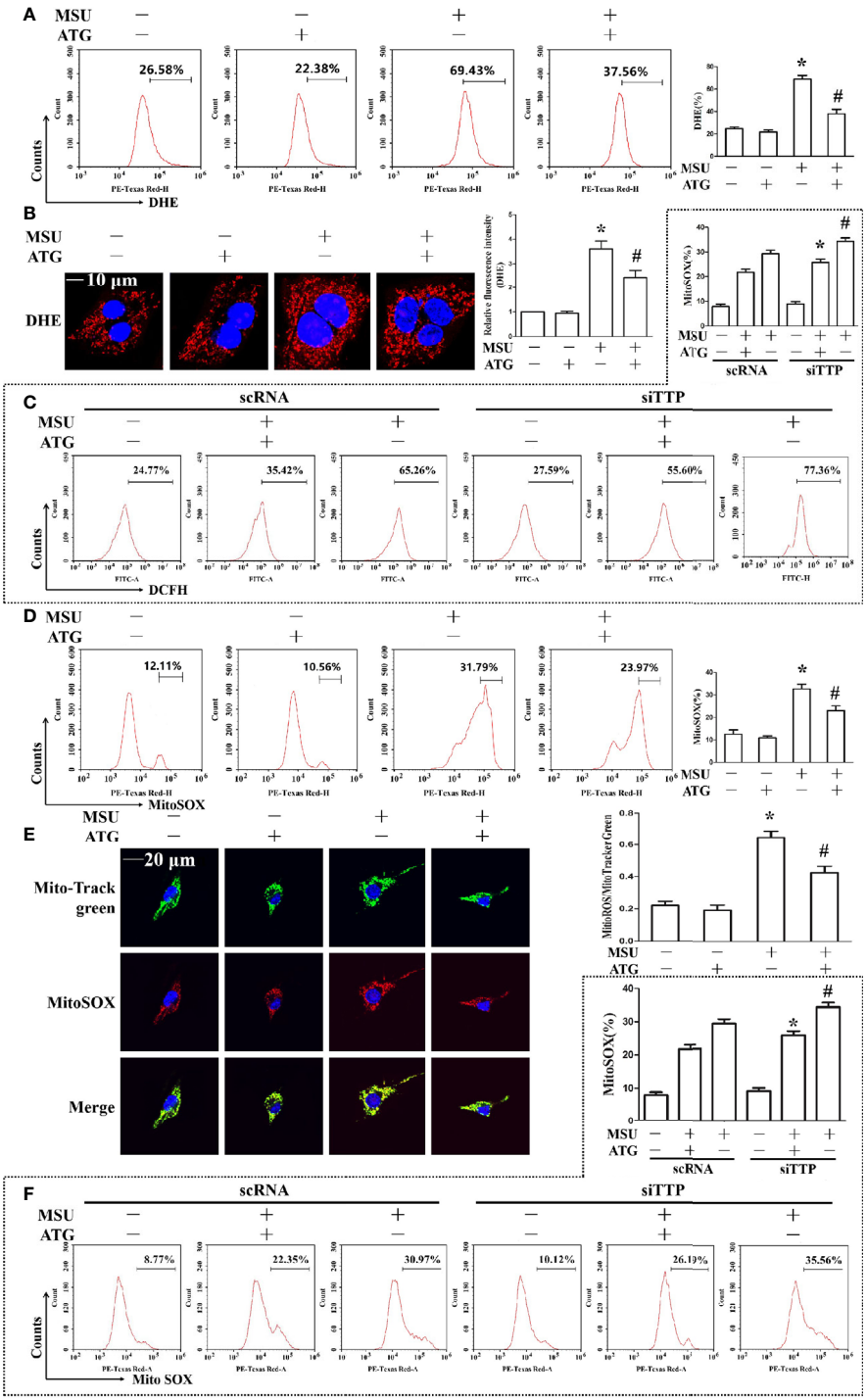


**FIGURE 5** | Effects of ATG administration on the MSU crystal-induced production of intracellular total ROS, mitochondrial ROS and mitochondrial membrane potential. **(A, B, D, E)** J774A.1 cells were pretreated with ATG (5 μM) for 1 h, primed with LPS for 1 h and then treated with MSU crystals (50 μg/ml) for 9 h. *P < 0.05 *vs.* without MSU crystals treatment; #P < 0.05 *vs.* MSU crystals treatment +vehicle. **(C, F)** J774A.1 cells were either transfected with scRNA or siTTP for 48 h, treated with 5 μM ATG for 1 h, primed with LPS for 1 h, and then stimulated with MSU crystals (50 μg/ml) for 9 h. *P < 0.05 *vs.* MSU crystals treatment + ATG + scRNA; #P < 0.05 *vs.* MSU crystals treatment + scRNA. **(A, B)** Cells were stained with DHE fluorescent probe, then the fluorescence intensity of DHE was measured by FACS or images were captured by Laser confocal microscope. Scale bar: 10 μm. **(C)** The fluorescence intensity of DCFH was measured by FACS. **(D)** Cells were stained with MitoSOX and then FACS was used to detect the fluorescence intensity of MitoSOX. **(E)** Cells were stained with MitoSOX and Hoechst 33342. Laser confocal microscope is used for image capture and fluorescence intensity analysis. Blue shows nuclei stained with Hoechst 33342. Scale bar: 20 μm. **(F)** FACS was used to detect the fluorescence intensity of MitoSOX. All the data are expressed as the means ± SEM from n=3 independent experiments.

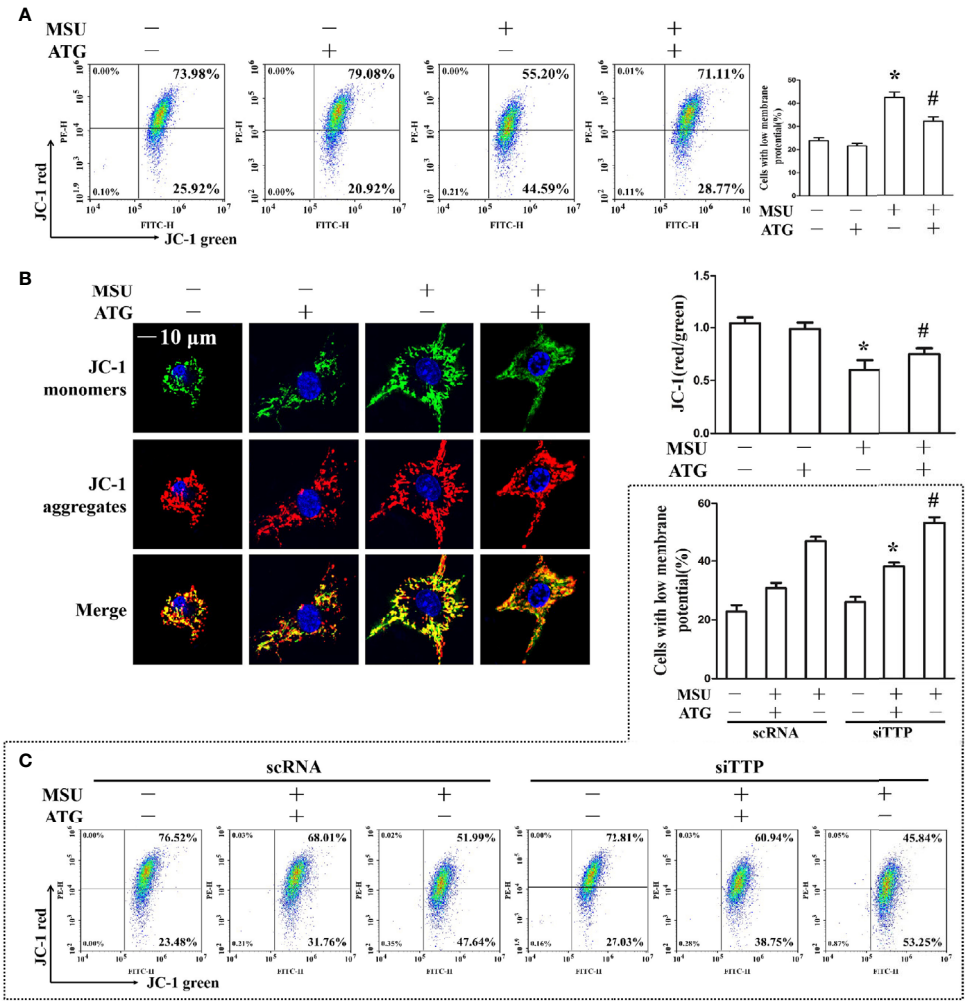


**FIGURE 6** | **(A, B)** J774A.1 cells were pretreated with ATG (5 μM) for 1 h, primed with LPS for 1 h and then treated with MSU crystals (50 μg/ml) for 9 h. *P < 0.05 *vs.* without MSU crystals treatment; #P < 0.05 *vs.* MSU crystals treatment +vehicle. **(A)** Cells were stained with JC-1 probe and FACS was used to measure mitochondrial membrane potential. **(B)** Cells were stained with JC-1 probe and Hoechst 33342. Laser confocal microscope was used to capture the fluorescence image and analyze fluorescence intensity. Blue shows nuclei stained with Hoechst 33342. Scale bar: 10 μm. **(C)** J774A.1 cells were either transfected with scRNA or siTTP for 48 h, treated with 5 μM ATG for 1 h, primed with LPS for 1 h, and then stimulated with MSU crystals (50 μg/ml) for 9 h. Cells were stained with JC-1 probe and FACS was used to measure mitochondrial membrane potential. *P < 0.05 *vs.* MSU crystals treatment + ATG + scRNA; #P < 0.05 *vs.* MSU crystals treatment + scRNA. All the data are expressed as the means ± SEM from n=3 independent experiments.

the MSU crystal-treated cells compared with the control group. Arctigenin treatment effectively alleviated the decrease in the mRNA expression levels of SOD1, SOD2 and CAT caused by MSU crystals but had little effect on GPX1 expression (**Supplementary Figure 5B**). These data suggest that Arctigenin treatment can prevent the imbalance in antioxidant defense system of J774A.1 cell caused by MSU crystals.

## Arctigenin Inhibits Lysosomal Rupture, Accelerates Lysosomal Biogenesis and Facilitates Autophagic Flux

Lysosomal dysfunction is closely associated with the activation of the NLRP3 inflammasome (22). It has been reported that lysosomal instability results in a decrease in the fluorescence intensity of the LysoTracker dye (23). In our study, MSU crystal-treated cells showed partial loss of the fluorescence signal of LysoTracker dye, while Arctigenin treatment abrogated the decrease in the fluorescence signal (**Figure 7A**). TTP knockdown hindered the regulation of ATG on lysosomal number (**Figure 7B**). Acridine orange produced red fluorescence when it accumulates within the lysosome and green fluorescence when it is released from ruptured lysosomes and diffuses into the cytoplasm and nucleus (24). Arctigenin treatment alleviated the decrease of red fluorescence signal induced by MSU crystals in the cytoplasm (**Figure 7C**), but in TTP knockdown cells, this function of ATG is disturbed (**Figure 7D**). Arctigenin treatment inhibited the increase in green fluorescence caused by MSU crystals (**Figure 7E**), TTP knockdown yet impeded this function of ATG (**Figure 7E**).

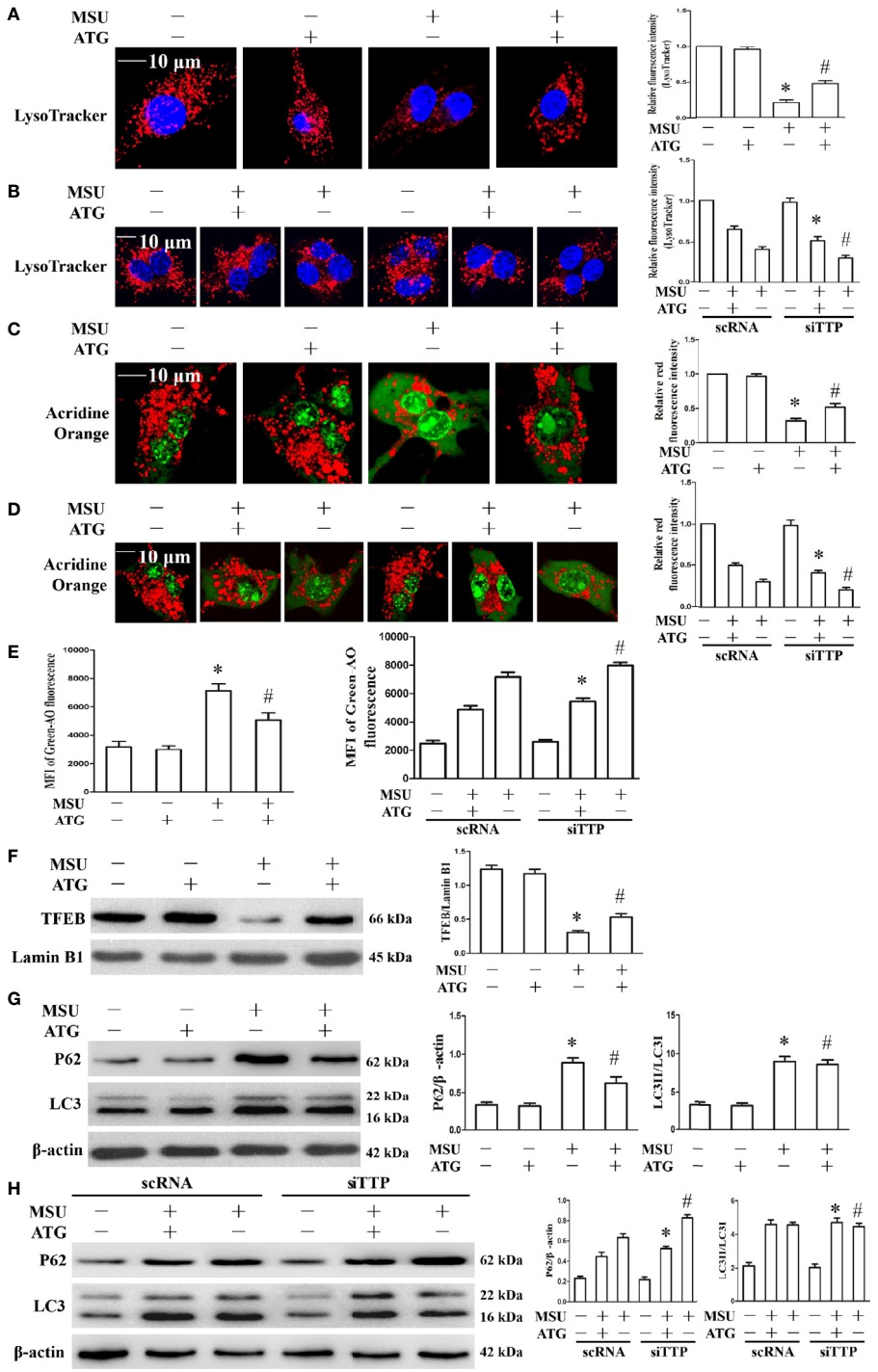


**FIGURE 7** | Effects of ATG treatment or TTP knockdown on MSU crystal-induced lysosomal rupture, lysosomal biogenesis and autophagic flux in J774A.1 cells. **(A, C, E–G)** J774A.1 cells were pretreated with ATG (5 μM) for 1 h, primed with LPS for 1 h and then treated with MSU crystals (50 μg/ml) for 9 h. *P < 0.05 *vs.* without MSU crystals treatment; #P < 0.05 *vs.* MSU crystals treatment +vehicle. **(B, D, H)** J774A.1 cells were either transfected with scRNA or siTTP for 48 h, treated for 1 h with 5 μM of ATG, primed with LPS for 1 h, then stimulated with MSU crystals (50 μg/ml) for 9 h. **(A, B)** The cells were stained using LysoTracker Red (100 nM, 30 min) and then with Hochest 33342 (20 μg/ml, 15 min). Images were captured by Laser confocal microscope. Scale bar: 10 μm. **(C–E)** cells were incubated with AO for 30 min, and then images were captured by Laser confocal microscope or green fluorescence intensity of AO was determined by a microplate reader. Scale bar: 10 μm. **(F)** Western blot analysis of TFEB level in nuclear proteins. **(G, H)** Western blotting was used to measure the LC3II and P62 protein levels. The LC3II and P62 protein levels were measured by Western blot assay. *P < 0.05 *vs.* MSU crystals treatment + ATG + scRNA; #P < 0.05 *vs.* MSU crystals treatment + scRNA. All the data are expressed as the means ± SEM from n=3 independent experiments.

These findings imply that ATG mitigates the MSU crystal-induced increase in lysosomal membrane permeability in a TTP-dependent manner.

TFEB (transcription factor EB) is a master transcription regulator of a subset of genes involved in lysosomal biogenesis and autophagy (25, 26). After MSU crystal treatment, the TFEB protein level in the nucleus was significantly reduced. Importantly, Arctigenin treatment counteracted the impairment of the TFEB protein level in the nucleus (**Figure 7F**). Next, we explored whether Arctigenin could induce lysosomal biogenesis, which is another potential reason for the increase in the red signal of LysoTracker staining, so we determined the effect of Arctigenin on the expression of lysosomal marker genes (Lamp1, Lamp2, Ctsb, and Ctsd). Quantitative RT-PCR data indicated that MSU crystal stimulation inhibited the mRNA expression of Lamp1 and Lamp2, and had little influence on the mRNA expression of Ctsb and Ctsd (**Supplementary Figure 6**). However, after Arctigenin pretreatment, Lamp1, Lamp2, Ctsb and Ctsd were all significantly elevated at the mRNA level (**Supplementary Figure 6**). Previous studies have shown that although MSU crystals can increase the expression of LC3-II, a marker for autophagosome formation, autophagic flux is blocked (20, 27). Arctigenin had no effect on the expression of LC3-II induced by MSU crystal, but significantly attenuated the expression of P62 induced by MSU crystals (**Figure 7G**). The results indicated that Arctigenin might promote autophagic flux and thus prevent p62 aggregation induced by MSU crystals. It has been confirmed that TTP plays an important role in autophagic flux and is necessary to form autophagolysomes and to clear pathogens (28). As shown in **Figure 7H**, by detecting LC3II and P62 protein levels, our data showed that Arctigenin-induced autophagic flux decreased in the TTP knockdown and MSU crystal-treated J774A.1 cells compared with the scRNA transfected and MSU crystal-treated J774A.1 cells. These results suggest that TTP is necessary for Arctigenin-mediated autophagic flux.

## Arctigenin Ameliorates MSU Crystal-Induced Peritoneal Inflammation and Arthritis *In Vivo*

MSU crystal deposition is the major cause of gouty arthritis. In the study, MSU crystals were respectively injected into the foot pad and into the abdominal cavity to induce arthritis and peritonitis to mimic the gout arthritis model. To confirm the role of Arctigenin in MSU crystal-induced inflammation *in vivo*, peritonitis model in C57BL/6 mice was used to evaluate the effect of Arctigenin on inflammatory cell influx and IL-1β production. The numbers of leukocytes, macrophages, neutrophils and basophilic granulocyte in the peritoneal fluid were assessed by staining the cells for the leukocyte marker CD45, the macrophage marker F4/80, the neutrophil marker Gr-1 and basophilic granulocyte marker CD63. As shown in **Figure 8A**, MSU crystals facilitated the infiltration of leukocytes, macrophages and neutrophils into the peritoneal cavity, but this was greatly suppressed by Arctigenin treatment (**Figure 8A**), there was little effect on the number of basophilic granulocytes (**Supplementary Figure 7**. Compared to vehicle treatment, Arctigenin treatment also greatly abrogated IL-1β secretion in peritoneal lavage fluids induced by MSU crystals (**Supplementary Figure 8A**).

Injection of MSU suspensions into the foot pad of mice can cause inflammation. Our data revealed that Arctigenin treatment relieved MSU crystal-induced swelling of the foot pad (**Figure 8B**). We measured IL-1β mRNA levels in foot pad tissue lysates and observed that Arctigenin dramatically blocked the expression of IL-1β (**Supplementary Figure 8B**). Histological analysis displayed obvious inflammatory cell infiltration in the sections of the foot pad tissue injected with MSU suspension (**Supplementary Figure 8C**), but Arctigenin administration visibly reduced the number of infiltrated leukocytes. We further confirmed the effect of Arctigenin administration on MPO positive cell distribution, Ly-6G$^+$ neutrophil and CD68$^+$ macrophage infiltration in foot pad tissue sections through immunofluorescence. The results showed that Arctigenin treatment prevented both neutrophils and macrophages from infiltrating into the foot pad tissue injected with the MSU suspension (**Figure 8C**) and blocked the protein levels of COX-2 and iNOS in the foot pad tissue injected with the MSU suspension (**Figure 8D**). Thus, these findings suggest that Arctigenin may be a potential candidate for the treatment of gouty arthritis.

# DISCUSSION

It has been reported that TTP is a negative regulator of many proinflammatory factors and is strongly expressed in active inflammatory sites, including RA synovial lining cells (29, 30). The major targets of TTP are the mRNA transcripts of cytokines. The TTP protein exists in two forms, the phosphorylated form, which is inactive, and the unphosphorylated form, which is active and induces mRNA decay. Thus, when TTP is phosphorylated, cytokine expression is upregulated, but when TTP is unphosphorylated, the production of target cytokines is inhibited. Unphosphorylated TTP is less stable and is easily degraded by the UPS (31, 32). Moreover, several studies have revealed that PP2A agonists may promote an increase in anti-inflammatory TTP activity (11, 12, 33). All of these findings prompted us to explore the effects of targeting TTP expression or promoting PP2A functional activity on the MSU crystal-induced inflammatory response.

In this study, we showed that exposure to MSU crystals accelerated the expression of TTP both *in vitro* and *in vivo*, but we did not investigate the underlying molecular mechanism by which MSU crystals promote TTP expression. It has been reported that IL-1β, TNF-α, IL-6, COX-2 and iNOS are targets of TTP-induced mRNA degradation (34, 35), and these inflammatory factors are also involved in MSU crystal-induced inflammation. Our data indicated that TTP knockdown led to elevated mRNA and protein levels of these inflammatory factors in macrophages stimulated by MSU crystals. The NLRP3 inflammasome is the main pathway of MSU crystal triggering cell inflammatory response. Haneklaus et al. reported that TTP is a crucial negative regulator of the NLRP3 inflammasome (17), and consistent with this report, we also observed that TTP knockdown promoted NLRP3 expression at the posttranscriptional level and NLRP3 inflammasome activation induced by MSU crystals.

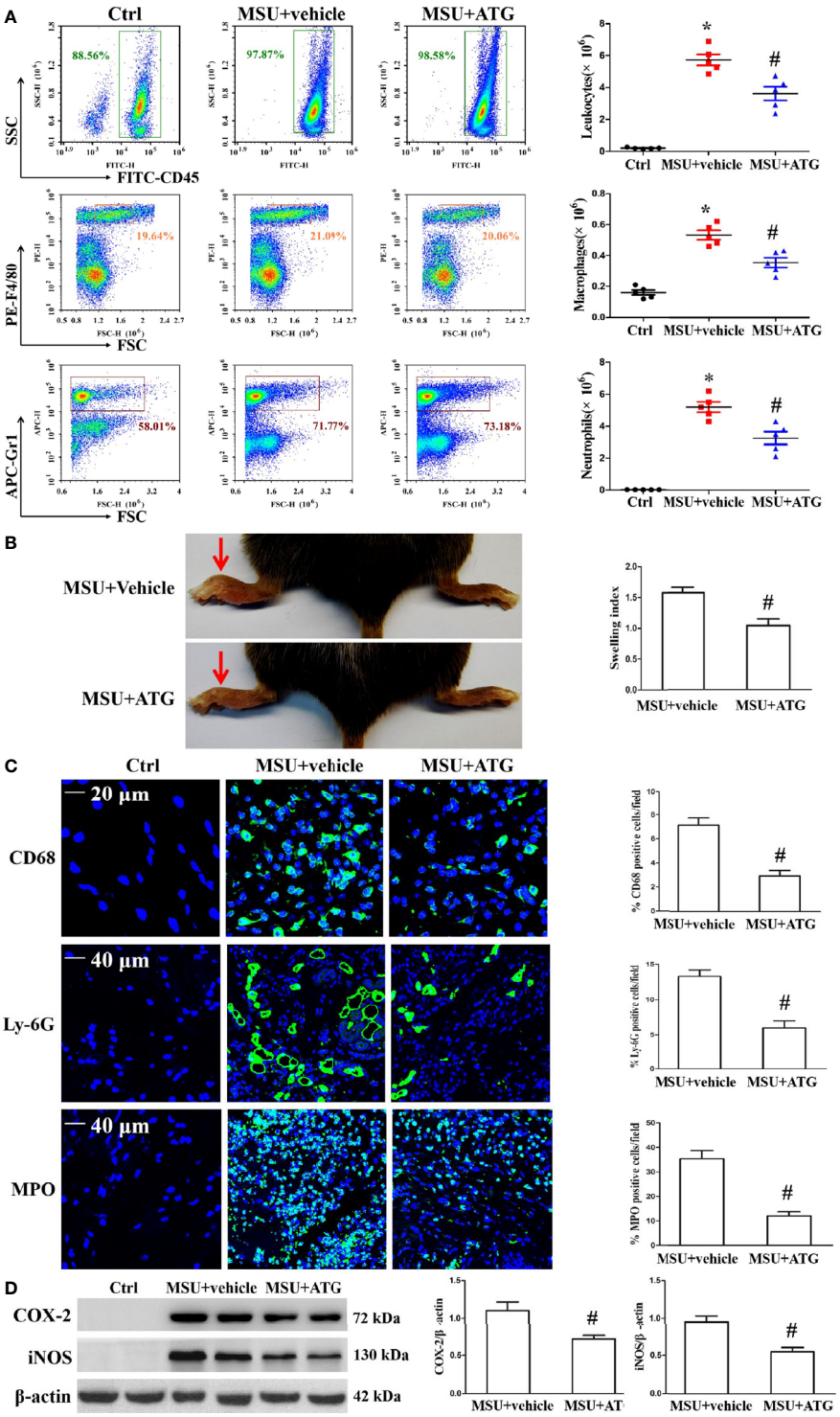


**FIGURE 8** | Effects of ATG on MSU crystal-induced inflammatory cell infiltration and cytokine release in a mouse model of peritonitis and arthritis. **(A)** After the cells precipitated from the peritoneal fluid and were respectively stained with FITC-CD45 Ab, PE-F4/80 Ab, and APC-(Ly-6G and Ly-6C) Ab. The percentage and cell numbers of migrated leukocytes, macrophages, and neutrophils were analyzed using FACS. **(B)** Paw swelling index. **(C)** An immunofluorescence assay was used to detect macrophage, neutrophil and MPO positive cells distribution in mouse foot pad tissue sections. Blue shows nuclei stained with Hoechst 33342. **(D)** Western blot assays were used to measure the protein levels of COX-2 and iNOS in foot pad tissue. n=5 for each group, *P < 0.05 *vs.* Ctrl; #P < 0.05 *vs.* MSU crystals + vehicle. All the data are expressed as the means ± SEM from n=3 independent experiments.

PP2A is a phosphatase that can dephosphorylate TTP (36) and is closely associated with TTP activation. To date, the best known PP2A activator is the sphingosine analog FTY720, FTY720 has other roles, most notably as a functional antagonist of the S1P pathway. Previous study has shown that the active metabolites of FTY720 can activate PP2A and inhibit the production of IL-1β induced by MSU crystals (37), but the molecular mechanism of its inhibition of IL-1β production has not been thoroughly explored. Arctigenin is a natural product, so we chose to study the effect of ATG on the inflammatory response induced by MSU crystals and its molecular mechanism. This study showed that MSU crystals promoted the activity of PP2A and that PP2A agonist inhibited the inflammation induced by MSU crystals *in vitro* and *in vivo*. Previous studies have reported that Arctigenin can alleviate the activity of NLRP3 inflammasome (38, 39). Our data showed that Arctigenin augmented PP2A activity, ameliorated inflammatory factors expression and NLRP3 inflammasome activation in J774A.1 cells stimulated by MSU crystals. More importantly, Arctigenin may mediate its anti-inflammatory effects in a TTP-dependent manner. Mitochondrial dysfunction is considered to be an important factor triggering the activation of the NLRP3 inflammasome (38), and mtROS overproduction is a key factor in NLRP3 inflammasome activation (39). It has been reported that Arctigenin can exert antioxidative effects and enhance the activities of antioxidant enzymes (21). We found that MSU crystal treatment led to a significant increase in intracellular and mitochondrial ROS, and Arctigenin treatment reduced the ROS production induced by MSU crystals, suggesting that the mechanism by which Arctigenin inhibits the activation of the NLRP3 inflammasome may be related to the reduction of ROS production. Arctigenin-mediated inhibition of mitochondrial ROS production is beneficial for improving mitochondrial function. Mitochondrial dysfunction is closely related to the loss of mitochondrial membrane potential. In this study, we found that Arctigenin inhibited the decline in mitochondrial membrane potential. Our study for the first time shows that TTP plays an important role in regulating mitochondrial dysfunction induced by MSU crystals and ATG regulates mitochondrial dysfunction in a TTP-dependent way, which is of great significance for further studies on the anti-inflammatory molecular mechanisms of ATG and TTP. It has been reported that ROS can regulate lysosomal membrane stability (40). In our study, Arctigenin also relieved lysosomal membrane stability, which is a crucial upstream regulatory factor for NLRP3 activation (41). More importantly, TTP knockdown impeded the protective function of ATG on lysosomes. These data suggest that Arctigenin may protect lysosomal membrane stability by alleviating mitochondrial oxidative stress in a TTP-dependent manner.

As the NLRP3 inflammasome is the main pathway in the response to MSU crystals, strategies that inhibit its activation or affect its activity can relieve gouty inflammation. Although TTP has been reported to play an important role in many inflammatory responses, we investigated that TTP could not only modulate the expression of inflammation-related genes, but also regulate the cleavage of caspase-1 in NLRP3 inflammasome in MSU crystal-induced inflammation, which may have an important influence on the pathogenesis of GA. Treatment with Arctigenin effectively inhibited the inflammatory response induced by MSU crystals in animal models of peritoneal inflammation and arthritis. Our data indicated that Arctigenin could both improve mitochondrial function and promote autophagy flux in a TTP-dependent manner; it would be

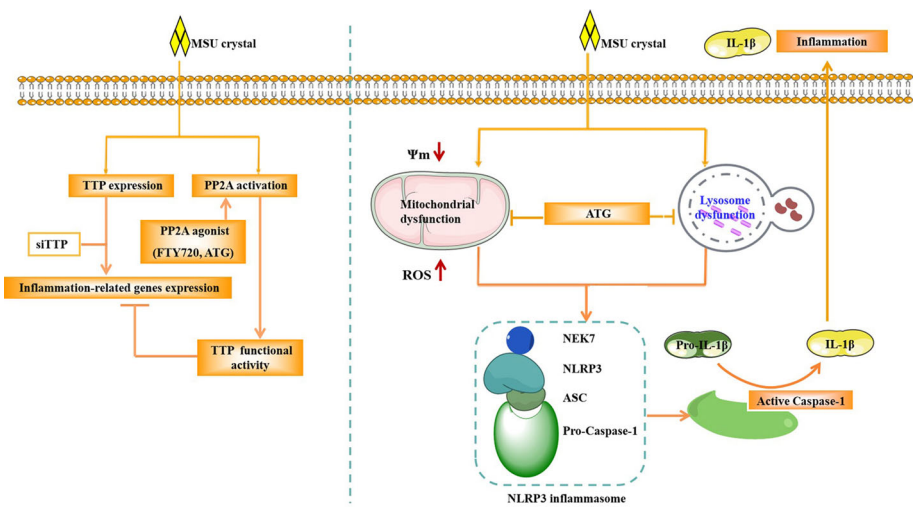


**FIGURE 9** | Schematic of the signaling pathway involved in TTP and PP2A activity in the MSU crystal-induced inflammatory response. After MSU crystal stimulation, TTP expression and PP2A activity were increased. TTP knockdown promoted the expression of inflammation-related genes induced by MSU crystals. However, PP2A agonist (ATG) treatment relieved the expression of inflammation-related genes induced by MSU crystals. ATG may alleviate the production of mitochondrial reactive oxygen species, increase mitochondrial membrane potential and improve lysosomal function, thereby inhibiting the activity of NLRP3 inflammasome, and ultimately reducing the inflammation caused by MSU crystals.

interesting to further investigate how Arctigenin regulates the relationship between mitochondrial autophagy induced by MSU crystals and the activation of NLRP3 inflammasome.

In Conclusion, our study demonstrated that TTP can regulate the expression of inflammation-related gene and the activity of NLRP3 inflammasome in response to MSU crystals (**Figure 9**). PP2A agonist can mitigate inflammatory response induced by MSU crystals through by improving lysosome and mitochondrial function, thereby inhibiting the activation of NLRP3 inflammasomes (**Figure 9**). The anti-inflammatory function of the PP2A agonist (Arctigenin) might be associated with TTP activation in MSU crystal-induced inflammation.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee of North Sichuan Medical College.

## AUTHOR CONTRIBUTIONS

YX and MZ initiated and designed this study. FC and HJ performed and analyzed the majority of the experiments. LL, TQ, and QH wrote the manuscript. FL and LR performed and analyzed the individual experiments. JL and YX performed data curation. MZ supervised the study. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.675534/full#supplementary-material

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REVIEW
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# Beyond the Extra Respiration of Phagocytosis: NADPH Oxidase 2 in Adaptive Immunity and Inflammation

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Reactive oxygen species (ROS) derived from the phagocyte NADPH oxidase (NOX2) are essential for host defence and immunoregulation. Their levels must be tightly controlled. ROS are required to prevent infection and are used in signalling to regulate several processes that are essential for normal immunity. A lack of ROS then leads to immunodeficiency and autoinflammation. However, excess ROS are also deleterious, damaging tissues by causing oxidative stress. In this review, we focus on two particular aspects of ROS biology: (i) the emerging understanding that NOX2-derived ROS play a pivotal role in the development and maintenance of adaptive immunity and (ii) the effects of excess ROS in systemic disease and how limiting ROS might represent a therapeutic avenue in limiting excess inflammation.

**Keywords: NOX2, ROS, CGD, oxidative stress, systemic inflammation**

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## 1 INTRODUCTION

### 1.1 Reactive Oxygen Species

Reactive Oxygen Species (ROS) are small molecules that are derived from molecular oxygen. They can either be classed as radicals or non-radicals, depending on whether they have an unpaired electron (1). Superoxide (a radical) is typically restricted to the endosomal compartment, and can be converted into hydrogen peroxide ($H_2O_2$; a non-radical) at low pH. $H_2O_2$ can diffuse across membranes to oxidise specific targets, or can be converted to $O_2$ and $H_2O$ (2).

$H_2O_2$ is a very useful signalling molecule because it can be rapidly generated and rapidly removed *via* specific enzymes such as catalase, superoxide dismutase and peroxiredoxin enzymes. It can also be quenched by non-enzymatic means such as glutathione (GSH) (3). As such, by predominantly facilitating cysteine and methionine oxidation, $H_2O_2$ is integral to regulating several crucial facets of the immune response.

ROS are produced during metabolic reactions within many cellular compartments, including the mitochondria, peroxisome and endoplasmic reticulum (4, 5). This review, however, will focus on ROS specifically produced in the phagosomes and at the cell membrane, by the phagocyte NADPH oxidase NOX2. ROS generation can occur from many sources in cells. These include mitochondria, peroxisomes and the P450 enzyme system. The NADPH oxidase is the first example of an enzyme where generating ROS is the primary function of the system, not a by-product of another process, e.g. the generation of ATP in mitochondria [discussed in (6)].

## 1.2 A Brief History of ROS Discovery

The physiological production of ROS was first described in 1908, by the German biochemist Otto Warburg, who identified that following the fertilisation of sea urchin eggs, $H_2O_2$ production succeeded a large and rapid increase in oxygen consumption (7). He suggested the existence of a respiratory enzyme that utilised oxygen to generate ROS, for which he won the Nobel Prize in Physiology and Medicine (8).

The ability of phagocytes to produce ROS was first noted by Baldridge and Gerrard in 1933 who described a marked increase in oxygen uptake by canine neutrophils following phagocytosis (9). Sbarra and Karnovsky extended these findings to show that "this burst of extra respiration" was accompanied by glucose consumption *via* the hexose monophosphate shunt and lactate production (10). Crucially, inhibitors of mitochondrial respiration have no effect on the oxygen consumption that accompanies phagocytosis. This is because the purpose of the oxygen consumption is independent from aerobic glycolysis, and is instead required to generate ROS.

Further key milestones followed, including (i) the finding that NADPH is the dominant physiological electron donor (although both NADH and NADPH can act in this capacity) that allows the production of ROS (11–13) and (ii) the seminal observation that the process starts with the generation of superoxide (14).

These findings show that neutrophils possess an enzyme that facilitates the donation of electrons to molecular oxygen. The identification of cytochrome b558, which we refer to as NOX2, as the relevant enzyme resulted from insightful biochemistry and the study of the monogenic immunodeficiency X-linked chronic granulomatous disease (X-CGD). This "fatal granulomatous disease of childhood" was first described in the 1950s. It described boys whose neutrophils were unable to kill certain bacteria and did not increase oxygen consumption or produce ROS (15).

In a landmark study for the field, Segal and colleagues showed that neutrophils from patients with CGD lacked both NADPH oxidase activity and a particular unusual b type cytochrome that localised to the plasma membrane (16, 17). The suspected causative genetic region was localised to Xp21 and cloned (18). The cDNA identified from such studies was used to make a translated protein and an anti-serum was raised to it. Elegant studies showed that the anti-sera stained a 91kDa protein found in "purified cytochrome b558" preparations. Crucially, it could not stain neutrophils from patients with X-CGD (19). Thus, the unusual cytochrome identified by Segal was indeed the product of the gene that was disrupted in X-CGD. However, it was clear that the story was not quite that simple. For instance (i) the 91kDa membrane-bound protein transcribed and translated from the X chromosome co-purified with a 22kDa protein (20, 21) and (ii) it transpired that there were autosomal recessive forms of CGD (AR-CGD) associated with a deficiency of other specific proteins (22, 23).

## 1.3 Chronic Granulomatous Disease

The phagocyte NADPH oxidase (NOX2) is a multi-subunit protein complex that, upon interaction, can form an active enzyme complex capable of producing superoxide. It is comprised of two integral membrane bound components; the 91kDa gp91*phox* and 22kDa p22*phox*, which together form cytochrome b558 (16, 21). p22*phox* binds to and stabilises gp91*phox*, preventing its degradation and its own in return. The cytosolic components comprise of p40*phox* (24), p47*phox* (22), p67*phox* (22) and Rac1 (25) or Rac2 (26). Following stimulation, p47*phox* becomes phosphorylated, allowing the complex to translocate to the membrane where it can associate with the gp91*phox*-p22*phox* heterodimer, forming the activated complex that transfers electrons from NADPH to molecular oxygen (**Figure 1**). This process is known as the respiratory burst, which is essential during the innate immune response (27–30). ROS can also be generated by the other NOX family members, NOX1, NOX3, NOX4, NOX5, DUOX1 and DUOX2 (28), however this review will focus on NOX2 derived ROS.

Our understanding of CGD has improved as several large-scale cohort studies have been conducted. X-linked CGD is predominant in Europe (31–33), the United States (34) and Japan (35), accounting for approximately 60% of cases. p47*phox* deficiency accounts for around 30% of cases and p22*phox* and p67*phox* deficiency for the remaining 10%. AR-CGD is predominant in cohorts from countries such as Iran (36) or Turkey (37), where consanguineous marriage is more prevalent.

X-CGD patients have a more severe disease course than patients with the AR-CGD, presumably because they lack all oxidase activity (31). Residual oxidase activity in neutrophils is linked to reduced disease severity and modest production of ROS seems to confer a greater likelihood of long-term survival.

## 1.4 Anti-Microbial Action of NOX2-Derived ROS

The role of NOX2-derived ROS was first identified in killing microbes. This is well illustrated by the susceptibility of patients with CGD to an extensive, but nonetheless demarcated, range of pathogens. For example, neutrophils from patients with CGD can kill *E. coli* but not *S. Aureus*. The focus of this review is the extra-phagosomal role of ROS, but it is worthwhile describing briefly how neutrophil-mediated killing takes place. This can be both direct, where hydrogen peroxide is likely more microbicidal than superoxide, but also indirect through integration with other systems. Here reactive oxygen species collaborate with granule proteins such as myeloperoxidase (MPO). In the presence of $H_2O_2$, MPO catalyses the production of Hypochlorous acid (HOCL), a very potent anti-microbial agent. HOCL, in turn, can modify multiple proteins (both host and microbe) in the phagosome to generate chloramines and aldehydes.

While we do not cover these processes in detail, we would direct readers interested in this fascinating area to excellent reviews by Nauseef (38) and also, Winterbourn and Kettle (39). These expand more on a few key points:

(i) That there is synergy between ROS and other granule constituents, including HOCL and non-oxidative killing mechanisms such as cathelicidins, serine proteases and lactoferrin.

(ii) That microbes employ a variety of strategies to evade phagosomal killing.

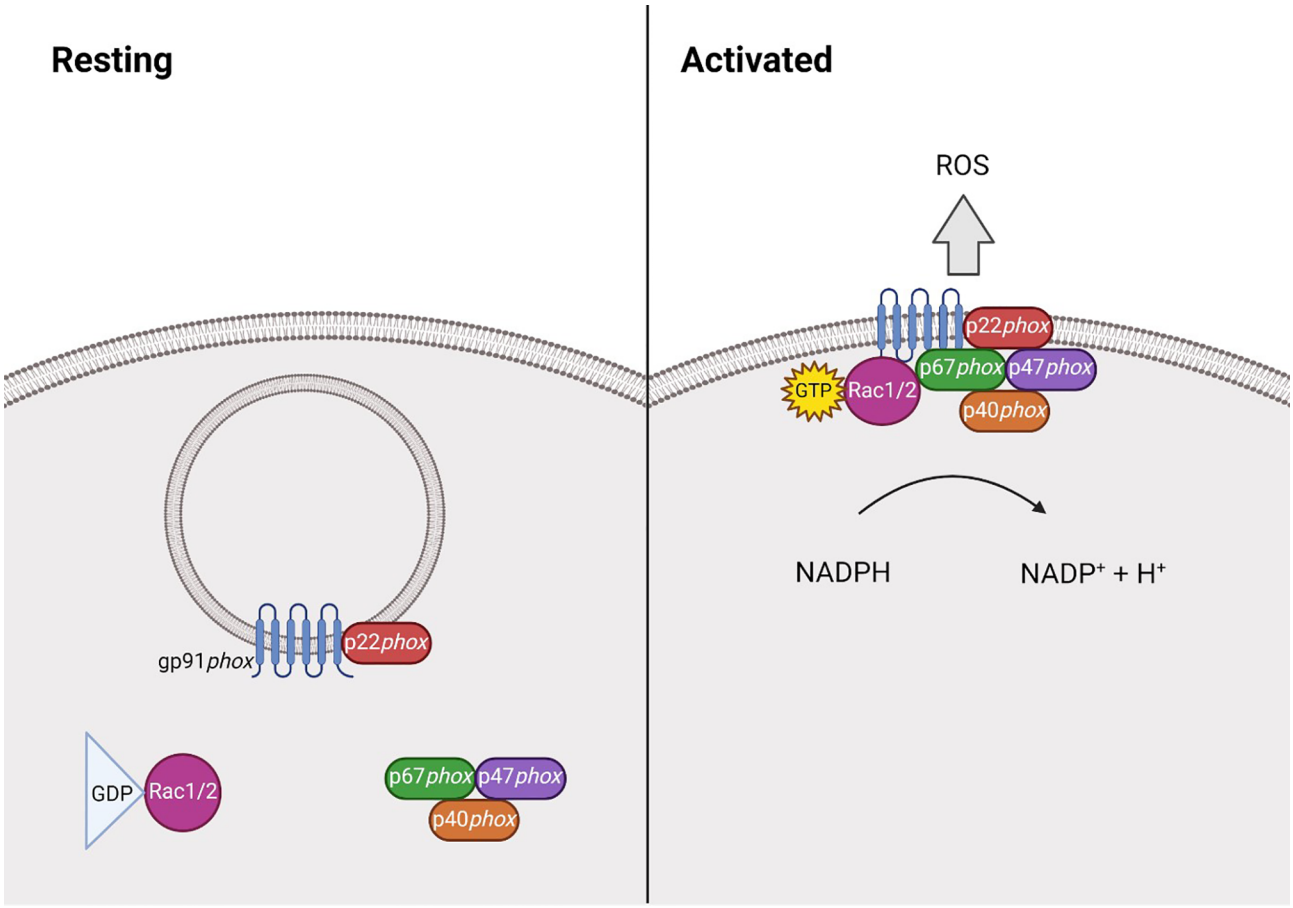


**FIGURE 1** | Structure of NADPH oxidase 2 during resting and activated states. At rest NADPH oxidase 2 (NOX2) is comprised of the membrane bound heterodimer gp91*phox*-p22*phox*, and the cytosolic component, comprised of p40*phox* (24), p47*phox* (22), p67*phox* (22) and the GTPase Rac1 (25) or Rac2 (26). Following stimulation, p47*phox* becomes phosphorylated and the Rac protein becomes GTP-bound, allowing the complex to translocate to the membrane where it can associate with the gp91*phox*-p22*phox* heterodimer. Together, this forms the activated complex that generates reactive oxygen species (ROS) by transferring electrons from NADPH.

(iii) That though the phagocyte NADPH oxidase is present in both neutrophils and macrophages, the intra-phagosomal environment in these cells differs greatly with respect to parameters such as pH and other anti-microbial components (such as MPO).

## 1.5 EROS Regulates the Expression of NOX2

An important recent addition to the biology of the phagocyte NADPH oxidase and CGD is the discovery of EROS (gene symbol CYBC1) (40). In 2017, we demonstrated that mice deficient in the previously uncharacterised open reading frame, bc017643, were exquisitely susceptible to infection with *Salmonella* Typhimurium and *Listeria monocytogenes*. It transpired that they failed to make ROS, owing to almost complete deficiency of gp91*phox* and p22*phox*. bc017643 encodes an ER-resident transmembrane protein that co-immunoprecipitates with gp91*phox*. We also observed that P2X7 receptor expression was downregulated in EROS deficient cells, which was later independently verified by another group (41), demonstrating that EROS mediates the expression of both gp91*phox* and P2X7. We characterised the protein encoded by bc017643 as EROS (Essential for Reactive Oxygen Species). Recent work in our laboratory suggests that EROS is a highly selective placeholder chaperone, binding to an "unsatisfied surface" on the gp91*phox* precursor and stabilising it until p22*phox* can bind (Randzavola, Mortimer et al., submitted). The lack of ROS and NOX2 expression in EROS-deficient mice, akin to that of gp91*phox*-/- mice, suggested that mutations in the human orthologue *C17ORF62* might lead to CGD. In 2018, we and another group reported separate homozygous mutations in *C17ORF62*, leading to EROS-deficiency, as a novel cause of chronic granulomatous disease (CGD5) (42, 43). *C17ORF62* has been re-named *CYBC1* (CYtochrome B Chaperone 1).

Mutations in EROS represent the first type of CGD to arise as a result of altered stability or folding of gp91*phox*. Although they have not yet been implicated in CGD, several other proteins are

capable of regulating gp91*phox* abundance. Negative Regulator of Reactive Oxygen Species (NRROS) was described in 2014 as an ER-resident protein that binds gp91*phox* and facilitates its degradation (44), its role appearing opposite to that of EROS. Similarly, the heat shock proteins, hsp90 and hsp70, also have opposing effects on gp91*phox* abundance, stabilising and degrading it respectively (45). The lamin beta receptor (LBR) has an intriguing role in regulating gp91*phox* and neutrophils that are LBR deficient show lower expression of gp91*phox* and generation of zymosan-induced ROS (46, 47).

Regulating gp91*phox* is one method of regulating ROS production. It is essential to tightly control the levels of ROS and subsequent sections of this review examine the tissue damage that can be caused by excess ROS production. However, too little ROS can also lead to inflammation. A key observation from studying CGD is that patients do not only experience opportunistic infections, but also present with autoinflammatory and autoimmune manifestations. These manifestations are characterised by sterile granulomatous inflammation, a hallmark of CGD (48). CGD patients often develop autoimmune diseases in which the pathogenesis is driven by autoantibody production, such as systemic lupus erythematosus and juvenile rheumatoid arthritis (31, 34). By producing $H_2O_2$, the phagocyte NAPDH oxidase regulates multiple pathways involved in innate anti-microbial defence, often serving to restrain inflammation in the process.

### 1.6 NOX2 Regulates Inflammation and Immune Signalling

The inflammatory manifestations that affect CGD patients arise as loss of ROS signalling impairs type 1 interferon signalling and autophagy (29). Patients with X-CGD are between 50-90% more likely to experience inflammatory episodes compared to patients with AR-CGD (49, 50), suggesting NOX2, particularly gp91*phox*, is essential for controlling the balance between a successful immune response and tissue damage.

## 2 NOX2 IN REGULATING PROCESSES IN ANTIGEN PRESENTATION

The regulation of type 1 interferon signalling, inflammasome activation and autophagy is well documented in the innate immune system and we have covered this in previous reviews (29, 30). The role of ROS in adaptive immunity starts with its pivotal role in antigen presentation.

### 2.1 MHC Class I Processing and Presentation

In normal circumstances, ROS generation occurs concomitantly with phagocytosis, regardless of cell type. Superoxide is a weak base and tends to alkalinise the phagosome, which influences proteolysis. A key study showed that the normal pH of dendritic cells (DCs) is neutral, tending towards slightly alkaline, but that NOX2-deficient DCs have acidic phagosomes which leads to enhanced antigen degradation and impaired cross presentation to CD8+ T cells *via* MHC Class I (51). The same group reported similar results in human DCs (52) and that Rac2 was key for NADPH oxidase assembly in CD8+ DCs (53). The small GTPase Rab27a was also necessary for NAPDH oxidase assembly (54). However another group, using slightly different conditions, found that while NOX2 did indeed reduce phagosomal proteolysis, this was not associated with significant changes in phagosomal pH. Rather, this group proposed that in DCs and macrophages, NOX2 affects proteolysis through reversible inhibition of the action of cysteine cathepsins *via* $H_2O_2$-driven oxidation of cysteine residues. Aspartic cathepsins are unaffected by the presence of NOX2 and thus the phagocyte NADPH oxidase was proposed to alter the activity of only a subset of proteases, skewing the peptide repertoire generated (55, 56).

A recent publication by Reis e Sousa and colleagues provided an intriguing new insight into the role of NOX2-derived ROS in antigen presentation (57). The DNGR1 receptor, expressed on the conventional DC1 (cDC1) subset of DCs, is essential for effective cross-presentation. DNGR1 binds F-actin on dead cell corpses, and has a short hemi-ITAM motif that can recruit and activate Syk. Mice that are deficient in DNGR1 or Syk expression in DCs have impaired cross presentation (58).

This group demonstrated that DNGR1 ligation facilitates Syk kinase activation and this, in turn, leads to NOX2 activation within phagosomes containing internalised antigen. The oxidative stress caused by the resulting free radicals damages the phagosome, causing membrane rupture, thus allowing leak of antigen into the cytosol and its translocation into the MHC class I presentation pathway. Cross presentation is markedly impaired in gp91phox-deficient DCs. How exactly Syk drives NOX2 activation in this context is unknown but the NOX2 activators Vav1 and Rac have previously been shown to be necessary for efficient cross-presentation and they are likely to be involved to some extent.

### 2.2 MHC Class II Processing and Presentation

There is also evidence that NOX2 can influence MHC class II peptide processing and presentation. Extending their previous work on cysteine cathepsins Yates and colleagues demonstrated that NOX2 not only affects the amount of proteolytic processing but affects it qualitatively too (59). They used the myelin oligodendrocyte glycoprotein (MOG) -induced model of experimental autoimmune encephalomyelitis (EAE) and showed that NOX2-derived macrophages (though interestingly not DCs) were defective in their ability to process and present the I-A(b)-immunodominant peptide of MOG. As such, p47*phox* or gp91*phox* deficient mice were partially protected from the central nervous system injury and inflammation that characterises EAE. MHC class II presentation is also impaired in human B cells deficient in p40*phox* (60). This was most marked for cytoplasmic and endogenous antigen but processing of membrane antigen was normal (see *Altered Humoral Immune Response in NOX2 Deficiency* below).

# 3 NOX2 IN REGULATING ADAPTIVE IMMUNITY

Although most commonly associated with innate immunity, NOX2 also has a variety of signalling roles in T and B cell responses. Some of these include; modulating T helper differentiation, proliferation of B cells and inducing apoptosis.

## 3.1 NOX2 in CD4+ T Cells

### 3.1.1 NOX2 Signalling Influences CD4+T Helper Differentiation

There are many conflicting studies regarding the influence of NOX2 on the differentiation of T helper subsets, summarised in **Table 1**. Briefly, the first published study on T helper differentiation in NOX2 deficiency describes a preferential Th1 response in $NOX2^{-/-}$ CD4+ T cells (61). Secretion of IFNγ is typical of a Th1 response, IL-4 and IL-5 of a Th2 response, and IL-17 and TGFβ of a Th17 response. Jackson et al. (61) found an increase in IFNγ secretion and decrease in IL-4 and IL-5 secretion following stimulation with anti-CD3. However, Kwon et al. found an increase in IL-4 secretion following anti-CD3 and anti-CD28 stimulation, indicative of a Th2 response in NOX2 deficiency (62). In opposition to both, Tse et al. (63) describe that a Th17 response develops in $NOX2^{-/-}$ CD4+ T cells following anti-CD3 and anti-CD28 stimulation. They found decreased IFNγ and IL-4 secretion, but increased IL-17 and TGFβ secretion (63). Although, this group used the NOD strain of mice that are renowned for their autoimmune phenotype, whereas the studies mentioned previously used the C57BL/6 strain, which may account for the difference in findings. Most studies find a combined Th1/Th17 response in NOX2 deficiency, with increased levels of IFNγ, IL-17 and their associated transcription factors T-bet and RORγt (64–66). Interestingly, Lee et al. (60) found that under their specific polarising conditions, the differentiation of all T helper subsets was elevated when NOX2 was absent, demonstrating that ROS are required to balance the development of T cell responses. The importance of NOX2 in CD4+ T helper differentiation remains to be clarified, but it appears that proinflammatory Th1/Th17 skewing is favoured. There are currently no published studies on the effect of NOX2 deficiency on the differentiation of Th9 or T follicular helper cells (Tfh). It would be both interesting and important to understand the entire T cell phenotype in the context of NOX2 deficiency.

### 3.1.2 NOX2 Signalling Affects Treg Differentiation and Activity

NOX2 is involved in the differentiation of other T cell types, including T regulatory cells (Tregs). One study found fewer peripheral CD4+CD25+ Tregs and decreased FOXP3 expression in $NOX2^{-/-}$ mice, indicating that NOX2 derived ROS also play a role in controlling the development of Tregs (65). However, a recent study found no decrease in Treg number or function in CGD patients, except in those with X-linked gp91*phox* deficiency (67). The authors suggest this may coincide with the fact that $gp91phox^{-/-}$ CGD patients have more inflammatory symptoms than those with mutations in other NADPH oxidase subunits (49, 67).

NOX2 is required for restraining the expression of the immune suppressive molecules on Tregs. The expression of CTLA-4, GITR, CD39 and CD73 is significantly greater on $gp91phox^{-/-}$ Tregs. Additionally, $gp91phox^{-/-}$ Tregs have increased NF-kB activation and greater FOXP3 expression. Subsequently, Tregs deficient in gp91*phox* have greater suppressive activity than wildtype control Tregs (68). Interestingly, $p47phox^{-/-}$ Tregs have poorer suppressive capabilities compared to their wildtype counterparts (69). This may relate to the functions of p47*phox* independent of phagocyte NADPH oxidase (70, 71).

### 3.1.3 NOX2 Is Required for T Cell Apoptosis

NOX2 is required for inducing cell-intrinsic apoptosis in activated T cells during the resolution of an immune response (72). Apoptosis of excess T cells after antigen clearance is

**TABLE 1** | Summary of studies describing T helper differentiation in NOX2 deficiency.

| Skew | Cytokine / transcription factor altered | Antigen | Strain | Gene deleted | Ref |
|---|---|---|---|---|---|
| Th1 | ↑ IFNγ<br>↓ IL-4 ↓ IL-5 | Anti-CD3 | C57BL/6 | p47*phox*<br>gp91*phox* | (56) |
| Th2 | ↑ IL-4 | Anti-CD3 + anti-CD28 | C57BL/6 | gp91*phox* | (57) |
| Th17 | ↓ IFNγ<br>↓ IL-4<br>↑ IL-17 ↑ TGFβ | Anti-CD3 + anti-CD28 | NOD | p47*phox* | (58) |
| Th1/Th17 | ↑ IFNγ<br>↑ IL-17<br>↓ IL-4 ↓ IL-5<br>↑ T-bet<br>↓ GATA-3 | Anti-CD3 + anti-CD28 | C57BL/6 | gp91*phox* | (59) |
| Th1/Th17 | ↑ IFNγ<br>↑ IL-17<br>↑ RORγt | PMA + ionomycin | C57BL/6 | gp91*phox* | (60) |
| Th1/Th17 | ↑ IFNγ<br>↑ IL-17 | *In vivo* OVA challenge<br>*In vitro* anti-CD3 + anti-CD28 | C57BL/6 | gp91*phox* | (61) |

essential to prevent an over exuberant immune response when responding to new and repeated antigenic challenges. gp91*phox*$^{-/-}$ T cells display significantly improved survival *in vivo* following cytokine deprivation. Greater antigen-specific proliferative responses are also observed when compared to wildtype controls, due to the larger pool of T cells that remain after the initial antigen challenge (72). This increased T cell survival may account for the differences in cytokine secretion discussed in section *NOX2 Signalling Influences CD4+T Helper Differentiation*.

## 3.2 NOX2 in CD8+ T Cells

### 3.2.1 NOX2 Signalling Can Affect CD8+ T Cell Responses

CD8+ T cell responses are critical to eliminate intracellular pathogen infections. In the absence of NOX2, mice are highly susceptible to *Trypanosoma cruzi* infection. There are fewer CD8+ T cells present at baseline in p47*phox*$^{-/-}$ mice, and these fail to proliferate in response to *T. cruzi* infection (73). Conversely, p47*phox*$^{-/-}$ CD8+ T cells have improved survival and mice experience reduced viral titres in response to lymphocytic choriomenigitis virus (LCMV) infection. The authors state this improved CD8+ T cell viral response may be due to less immunopathology that occurs in the absence of p47*phox* (74). Similarly, gp91*phox*$^{-/-}$ mice have reduced inflammation and viral titres in response to influenza infection, however there was no difference in CD8+ T cell populations *in vivo* or influenza-specific CD8+ T cell responses *in vitro* (75). Therefore, the influence of NOX2 on CD8+ T cell responses may be dependent upon pathogen type.

### 3.2.2 NOX2 Is Critical for CD8+ Treg Driven Immunosupression

NOX2 is utilised by CD8+ Tregs to enable a novel Treg mediated suppression of CD4+ T cells (76). CD8+ Tregs are thought to release exosomes containing NOX2, which is taken up by CD4+ T cells located in nearby T cell zones of secondary lymphoid organs. NOX2 derived ROS inhibits the phosphorylation of the T cell receptor (TCR) signalling molecules ZAP70 and LAT, inhibiting TCR signal transduction. CD8+ Tregs treated with the flavoenzyme inhibitor diphenyleneiodonium (DPI), gp91ds-tat or short hairpin RNAs targeting NOX2 are unable to upregulate NOX2 and subsequently are unable to supress CD4+ T cell activation (76).

## 3.3 NOX2 in B Cells

### 3.3.1 NOX2 Elicits Bacterial Killing in B Cells

Similar to innate immune cells but unlike T cells, peritoneal B cells can utilise NOX2 derived ROS to kill intracellular bacteria. NOX2$^{-/-}$ B cells from NOX2 deficient mice have a reduced ability to produce the ROS required to kill engulfed pathogens, and therefore have greater survival of bacteria within phagosomes (77).

### 3.3.2 NOX2 Signalling Restrains Proliferation of B Cells

Following B cell receptor (BCR) stimulation, NOX2 is responsible for generating the rapid initial production of ROS, whereas the later stages of ROS production are not NOX2 dependant. NOX2$^{-/-}$ mice fail to produce ROS immediately after BCR stimulation, but BCR proximal signalling and subsequent downstream signalling pathways are normal (78, 79). However, NOX2$^{-/-}$ B cells have been found to undergo enhanced cell cycle entry following BCR stimulation (79, 80). This suggests NOX2 has a role in negatively modulating ROS-driven BCR induced proliferation in B cells.

### 3.3.3 NOX2 Is Involved in B Cell Signalling

NOX2 derived ROS often acts as a second messenger during various signalling pathways. Tyrosine phosphorylation and IgM secretion is impaired following BCR or TLR4 stimulation in NOX2 deficient B cells. Accordingly, lentiviral induced expression of NOX2 components can restore signalling capabilities in NOX2 deficient cells following BCR stimulation (81). NOX2$^{-/-}$ B cells have increased expression of the Toll-like receptors (TLR) TLR7 and TLR9, and subsequently have greater responsiveness to TLR7/9 stimulation (82). These studies demonstrate that NOX2 can modulate BCR signalling in a number of ways.

### 3.3.4 NOX2 Regulates MHC Class II Antigen Presentation on B Cells

Presentation of exogenous antigens requires antigen uptake and processing in endosomal or lysosomal compartments to generate the peptides to be presented on MHC class II molecules [reviewed in (83)]. p40*phox*$^{-/-}$ B cells are less able to present exogenous antigen on their MHC class II. However, p40*phox*$^{-/-}$ B cells preferentially present self-membrane resident antigens, suggesting p40*phox* may skew epitope selection and have implications for CD4+ T cell activation (60).

### 3.3.5 Altered Humoral Immune Response in NOX2 Deficiency

NOX2 may have a role in the production of antibodies. NOX2$^{-/-}$ mice have greater antibody production following injection of collagen (84, 85) and challenge with UV-irradiated bacteria (86). Cachat et al. (88) found an increase in IgG1 and IgG2c production in NOX2$^{-/-}$ mice following ovalbumin injection. A later paper found NOX2$^{-/-}$ mice have increased production of IgA, IgG, IgG1, IgG2b and IgG3 levels following influenza A infection (88). The authors suggest that functional NOX2 activation during influenza A infection results in the suppression of antiviral cytokines, preventing the development of humoral immunity (88). Interestingly, there may be some differences between human and mouse. IgG1 levels are decreased whereas IgG2 levels are increased in CGD patient serum (87). CGD patients also have significantly increased levels of B cell activating factor (BAFF), a B cell survival factor, and subsequently have greater IgM levels compared to healthy controls (89). CGD patients have decreased numbers of influenza-specific peripheral memory B cells but increased numbers of nonconventional CD27- memory B cells compared to healthy controls (90, 91). Although, despite abnormal numbers of B memory cells, influenza specific memory B cell

responses remain comparable to healthy controls (90). Therefore, NOX2 is involved in inducing and maintaining the humoral immune response, however the specific role of NOX2 in human B cell responses needs to be investigated further.

# 4 THE DELETERIOUS ROLE OF ROS IN SYSTEMIC INFLAMMATION

In the sections above, we have seen that ROS are pivotal for both normal innate and adaptive immunity. We have also described how a lack of ROS in CGD can lead to autoinflammation and autoimmunity.

However, we have also seen how the generation of ROS must be tightly controlled and its generation can outstrip the capability of those systems that regulate it. Excess ROS can cause tissue damage in a variety of ways, causing protein and DNA damage and lipid peroxidation.

## 4.1 Oxidative Stress in Systemic Disease

Oxidative stress is well known to be a contributing factor in the development of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and Multiple Sclerosis. Dysregulation or overproduction of ROS leads to oxidative stress which is thought to disrupt immune homeostasis in the central nervous system (CNS) and promote prolonged neuroinflammation (92). NOX proteins are important generators of ROS in the CNS and NOX2 expression has been documented in the CNS in microglia, neurons and endothelial cells (93).

# 5 REACTIVE OXYGEN SPECIES IN THE CNS

## 5.1 Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterised by the progressive loss of motor neurons in the brain, brain stem and spinal cord. Disease progression is rapid, with a prognosis of only 2-5 years after diagnosis for most individuals (94).

Several studies have demonstrated that NOX2 contributes to disease progression in the $SOD1^{G93A}$ transgenic mouse, a common mouse model of ALS. NOX2 expression and activation was shown to be significantly upregulated in microglia in the spinal cord of $SOD1^{G93A}$ mice compared to controls (95). NOX2 expression was also found to be increased in the spinal cord of sporadic ALS patients. The authors demonstrated that NOX2 deletion in $SOD1^{G93A}$ transgenic mice prolonged survival and slowed disease progression, suggesting that NOX2 activity contributes to the degeneration of motor neurons and disease progression in ALS. Another study demonstrated that treatment of $SOD1^{G93A}$ mice with apocynin, a NOX inhibitor, also increased survival and slowed disease progression (96). The authors also demonstrated that SOD1 regulated RAC1/NOX2 dependent ROS generation in a redox-dependent manner. ALS-associated SOD1 mutants resulted in enhanced activation of RAC1/NOX2 and increased ROS production in both cell lines and the spinal cord of $SOD1^{G93A}$ mice.

However, in contrast to this, a recent study found that NOX2 inhibition did not extend survival in $SOD1^{G93A}$ mice (97). Deletion of NOX1 or NOX2 in $SOD1^{G93A}$ mice did not increase survival or influence microglia activation in this study. Treatment of $SOD1^{G93A}$ mice with the NOX inhibitors thioridazine and perphenazine did not significantly increase survival or prevent motor neuron degeneration. A study of the oxidative burst in granulocytes in the peripheral blood of ALS patients did not identify any significant difference in NOX2 activity between ALS patients and matched controls. However, patients with lower NOX2 activity were found to have a significant increase in survival (98). Therefore, whilst NOX2 has been shown to be upregulated in both mouse models and patients with ALS, the exact role of NOX2 in ALS pathogenesis remains unclear.

## 5.2 Multiple Sclerosis

Multiple sclerosis (MS) is a common neurodegenerative disorder characterised by inflammation and demyelination in the CNS. Microglial activation is hypothesised to play an important role in the pathogenesis of MS. NOX2 has also been shown to be upregulated in microglia in active lesions in MS patients (99).

The mouse experimental autoimmune encephalomyelitis (EAE) model is commonly used as a model to study MS. Recent evidence has demonstrated that disease severity is reduced in $NOX2^{-/-}$ mice after EAE induction. Lymphocyte and microglial infiltration in the CNS was also significantly decreased compared to heterozygous and wild-type controls. Interestingly, the authors showed that only immune infiltration in the CNS is reduced, as immune cell populations in peripheral tissues such as the spleen and cervical lymph node are similar in $NOX2^{-/-}$ and wild-type mice post EAE induction (100). Microglial activation was also decreased in $NOX2^{-/-}$ mice, with inflammatory cytokine and chemokine secretion levels in the CNS also decreased.

Another recent paper demonstrated that deletion of NOX2 in conventional DCs (cDCs) reduced disease severity and demyelination in an adoptive transfer model of EAE (101). Interestingly, the authors demonstrated that deletion of NOX2 in cDCs reduced accumulation and activation of autoimmune CD4+ T cells in the CNS in EAE mice, suggesting that NOX2 regulates CD4 infiltration. Deletion of NOX2 also abrogated LC3-associated phagocytosis and CD4+ T cell activation through reduced myelin antigen presentation. This study highlights an important role for NOX2 in promoting inflammation and demyelination in EAE mice. Therefore targeting NOX2-dependent ROS production may slow disease progression and provide therapeutic benefit for patients with MS.

## 5.3 Alzheimer's Disease

Oxidative stress and damage have also been hypothesised to play a role in Alzheimer's disease, although the role of NADPH oxidases remains unclear. As mentioned previously, microglia

play a vital immune role in CNS homeostasis through clearance of dead cells and debris. However, dysregulation of microglia can lead to prolonged neuroinflammation and the development of neurodegenerative disorders. Microglia have been shown to associate with and promote clearance of amyloid-β (Aβ) deposits in the early stages of Alzheimer's. However, in aging mice microglia appear to have a reduced ability to clear Aβ deposits and drive inflammation within the CNS (102).

NOX2 expression in microglia has also been hypothesised to play an important role in the pathogenesis of Alzheimer's disease. A recent study demonstrated high NOX2 expression in microglia and increased microglial infiltration in aged wild-type brains, compared to young mice (103). Interestingly, $NOX2^{-/-}$ aged mice had significantly less Aβ deposition and plaque formation compared to aged wild-type controls. ROS production was also much lower in $NOX2^{-/-}$ mice than in wild-type mice, indicating that ROS production in the aged mice was NOX2-dependent. The authors also investigated ROS production in human brain tissue, and found older individuals had higher levels of ROS production when compared to young controls. Stimulation of the BV2 microglial cell line with $A\beta_{42}$ peptide also resulted in significantly increased NOX2-depdendent ROS production, which could be inhibited using NOX2 inhibitors such as apocynin or Nox2tat. These results indicate that NOX2 may play an important role in the regulation of microglia and the microglial response to Aβ plaques and therefore it may be an important driver of the pathogenesis of Alzheimer's disease.

Whilst it is clear that oxidative stress is involved in aging and the development of neurodegenerative diseases, the precise mechanisms defining how aberrant NOX2-depdendent ROS production drives neuroinflammation require further investigation. In addition, it remains to be investigated whether targeting of NOX2 through the use of inhibitors would provide therapeutic benefit in neurodegenerative disorders.

## 6 THE ROLE OF REACTIVE OXYGEN SPECIES IN THE LUNG

ROS production by phagocytes plays a vital role in the innate immune response and the clearance of pathogens during infection. However, it is essential that the mechanisms which regulate ROS generation are tightly controlled. Failure to regulate the innate immune response results in excessive ROS production, or oxidative stress, which promotes inflammation. Oxidative stress and the resultant sustained inflammation can result in tissue damage, particularly in barrier sites such as the lung (104). Recent evidence has implicated excessive NOX2-derived ROS production in acute lung injury, particularly during influenza infection.

In 2006, Snelgrove et al. demonstrated that deletion of NOX2 in mice resulted in reduced viral load after infection with Influenza virus (105). $NOX2^{-/-}$ mice exhibited enhanced viral clearance, increased lung function and reduced lung damage when compared to wild-type mice. Increased macrophage and neutrophil infiltration into the airway epithelia was also observed. Another study by Vahlos et al. in 2011 also demonstrated that deletion of NOX2 resulted in reduced viral titres in mice infected with Influenza A virus (75). Apoptosis of lung alveolar epithelial cells was greatly reduced in lung tissue sections of $NOX2^{-/-}$ mice compared to wild-type mice. Interestingly, in contrast to the earlier findings by Snelgrove et al., the authors demonstrated that immune cell infiltration in the bronchoalveolar lavage fluid (BALF) was significantly decreased in $NOX2^{-/-}$ mice. However, the authors hypothesise that this could be due to sex differences between the mouse models used in the studies. Treatment of wild-type mice with the ROS inhibitor apocynin after Influenza A infection also significantly reduced macrophage and neutrophil infiltration and viral titres were reduced by 50%. These results indicate that NOX2 is driving inflammation and acute lung injury in response to Influenza A infection. Therefore, modulation of NOX2-dependent ROS production may provide therapeutic benefit and reduce lung damage in patients suffering from acute lung injury during infection.

A recent study identified that Influenza A infection drives the production of endosomal NOX2-dervied ROS in response to TLR7 stimulation by viral RNA (88). Endosomal ROS was also found to suppress cytokine secretion in a TLR7-dependent manner. Treatment with apocynin significantly increased IL-1β, TNF-α, IFN-β and IL-6 secretion in wild-type macrophages in response to imiquimod. Interestingly, the authors identified a single cysteine residue, Cys98, which is highly conserved and unique to TLR7, as a novel redox sensor. $TLR7^{-/-}$ macrophages transfected with a TLR7C98A mutant could not restore TLR7-dependent cytokine secretion. The authors hypothesise that ROS production by NOX2 may modify the Cys98 residue, resulting in reduced cytokine secretion and a dampened antiviral response (88). In Nox2 deficient mice infected with Influenza A virus, IL-1β and IFN-β secretion was significantly increased. Serum and BALF levels of IgG and IgA were also significantly increased compared to wild-type mice, indicating that NOX2-derived ROS can also suppress antibody production. These results indicate that ROS production can inhibit important antiviral responses, thereby reducing the host's ability to efficiently clear viral pathogens.

Recent evidence has also demonstrated that NOX2 can modulate Type I Interferon (IFN) production in response to bacterial infections. $NOX2^{-/-}$ mice infected with *Listeria monocytogenes* exhibited an increased bacterial load, whereas $Ifnar1^{-/-}$ mice infected with *L. monocytogenes.* had a reduced bacterial load (106). Deletion of NOX2 in $Ifnar1^{-/-}$ mice also resulted in a reduced bacterial load, indicating that NOX2 regulation of Type I IFN controls *L. monocytogenes* infection. The number of infection foci was increased in $NOX2^{-/-}$ mice, however lymphocyte migration to infection foci was decreased in a Type I IFN-dependent manner. Interestingly, the authors also demonstrated that NOX2 deficiency upregulates IL-10 expression, which is known to play an anti-inflammatory role during infection. These results suggest a novel antimicrobial role for NOX2 in controlling *L. monocytogenes* infection through modulation of the Type I IFN response.

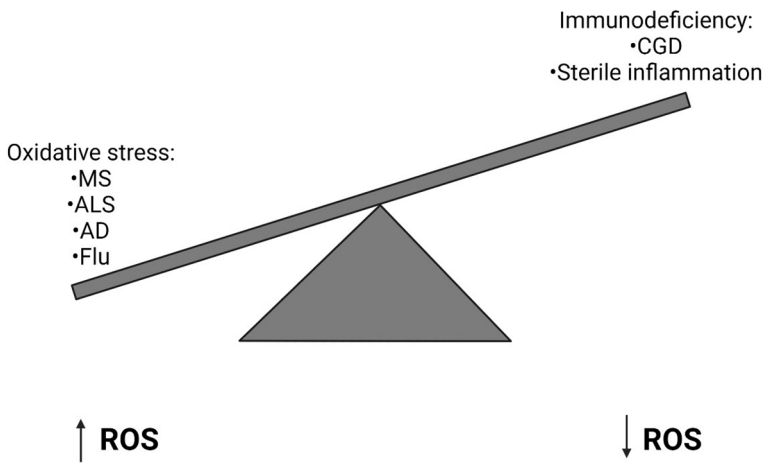


**FIGURE 2** | Imbalanced Reactive Oxygen Species generation can have implications for human health. High levels of Reactive Oxygen Species (ROS) can result in oxidative stress, which can lead to a number of diseases including Multiple Sclerosis (MS), Amyotrophic Lateral Sclerosis (ALS), Alzheimer's Disease (AD) and Influenza. Alternatively in primary immunodeficiencies, such as Chronic Granulomatous Disease (CGD), where a genetic defect means ROS are not generated, sterile inflammation often develops.

## 7 CONCLUSION

The balanced generation of ROS is essential to induce appropriate immune responses and avoid tissue damage by oxidative stress (**Figure 2**). ROS is required during the innate immune response to control invading pathogens and prevent infection. Too little ROS results in susceptibility to opportunistic pathogens, such as in CGD. However, increasing evidence has demonstrated that dysregulation of ROS production can result in sustained inflammation and tissue damage which can be fatal during severe infection. Recent evidence has also demonstrated that ROS can prevent antiviral immunity and reduce the body's ability to clear viral infections. Therefore, inhibition of NOX2 during infection may help to promote antiviral responses and prevent excessive ROS generation, thereby reducing the occurrence of acute lung injury, for example in Influenza infection. It is important to further investigate the mechanisms underlying the regulation of ROS generation and how they become dysregulated during infection in order to understand how they may be targeted for clinical benefit in the future. Beyond influenza A, the utility of blocking NOX2 action should be examined in other settings such as acute lung injury and Acute Respiratory Distress Syndrome (ARDS). The question of blocking host inflammatory pathways in infection related lung pathology has been shown into sharp focus by the COVID-19 pandemic where treatment with dexamethasone (107) or IL-6 inhibition (108) can have a beneficial effect when used at the correct stage of the disease. It will be interesting to see if NOX2 inhibition has any beneficial effect in either *in vitro* or animal models of COVID-19 as it is likely that the virus will continue to mutate and become endemic, possibly escaping vaccine-mediated control in some instances.

Numerous NOX2 inhibitors have been developed (109) and it is likely that advances in *in silico* technology such as alpha-fold will also better inform our efforts to inhibit the action of this and other NADPH oxidases (110, 111).

## AUTHOR CONTRIBUTIONS

PM, SM, and DT: conceptualisation and writing. DT: funding acquisition and final revision. All authors contributed to the article and approved the submitted version.

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# Hydrogen Peroxide Affects Growth of *S. aureus* Through Downregulation of Genes Involved in Pyrimidine Biosynthesis

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Reactive oxygen species (ROS) play a crucial role in the cellular defense against *S. aureus*, as evidenced by the importance of this pathogen in patients lacking the ROS-generating phagocyte NADPH oxidase NOX2. ROS concentrations required to kill *S. aureus in vitro* are much higher than those found in the phagosome. We therefore hypothesized that sublethal ROS concentrations may play a role in *S. aureus* gene dysregulation and investigated the *in vitro* transcriptomic response of *S. aureus* to sublethal concentrations of hydrogen peroxide ($H_2O_2$). A striking observation of these experiments was a coordinated and massive downregulation of genes involved in pyrimidine metabolism. Using transposon insertion mutants, we demonstrated that deletion of *carA*, a gene involved in pyrimidine synthesis, led to a significant growth defect and to an increased sensitivity of *S. aureus* to added $H_2O_2$. The phenotype of the *carA* mutant could be reversed through supplementation with the pyrimidine precursor uracil, or with a multicopy vector encoding *carA*. As opposed to the impact of ROS on extracellular survival, *carA* deletion did not affect the intracellular survival in neutrophils. Our results raise the possibility that ROS-dependent downregulation of pyrimidine metabolism might be a survival strategy of *S. aureus*, allowing colonization through intracellular survival, while decreasing the risk of killing the host through dampened extracellular growth.

**Keywords: *S. aureus*, hydrogen peroxide, pyrimidine metabolism, neutrophils, *carA***

## INTRODUCTION

*Staphylococcus aureus* is a Gram-positive, round-shaped facultative anaerobic bacterium, discovered in 1881 by Alexander Ogston, a Scottish surgeon. *S. aureus* colonizes approximately 30% of the human population (1) and its main site of colonization is the nasal cavity (2, 3). While *S. aureus* is generally commensal, it causes a broad spectrum of severe infections (4). *S. aureus* is able to adapt to

the environment (5) and, in the era of antibiotics, it has rapidly developed or acquired antimicrobial resistances. Development of new anti-staphylococcal treatments is now considered a high priority by the WHO (6).

Reactive oxygen species (ROS) generated by phagocytes are key players in the defense against *S. aureus*. This concept is largely based on the phenotype of chronic granulomatous disease (CGD), a genetic disorder caused by loss of function mutations in the ROS-generating phagocyte NADPH oxidase NOX2. CGD patients often suffer from severe, recurrent and/or persistent infections with *S. aureus* (7). Thus, in a simplistic model, NOX2-derived ROS are killing *S. aureus* by targeting DNA, proteins and lipids (8). However, *S. aureus* possesses a complex antioxidant defense system, including two superoxide dismutases and one catalase (9) and is quite resistant to microbicidal concentrations of ROS. The relationship between ROS and *S. aureus* might be more complex than a direct lethal effect on *S. aureus* and we hypothesized a more subtle mechanism that could affect the bacterial fitness.

Among the different ROS generated by NOX2, hydrogen peroxide ($H_2O_2$) may have a predominant role. High concentration of this non-radical oxidant can damage cells and tissues. However, based on available research, it is unlikely that these very high concentrations (>50 mM) can be reached within the phagosome as phagosomal $H_2O_2$ concentrations are in the micromolar range (10). However, there is increasing evidence that there are $H_2O_2$ gradients and therefore the local $H_2O_2$ concentration in close proximity to the NADPH oxidase might be higher and reach the low millimolar range (11). $H_2O_2$ is now increasingly recognized as an intra- and intercellular signaling messenger (12). $H_2O_2$ can impact cell phenotype through a variety of mechanisms, including regulation of gene expression (13). $H_2O_2$ reversibly oxidizes specific cysteine residues of key protein targets to regulate their function in eukaryotic cells (14). A similar effect of $H_2O_2$ on bacterial signaling was observed (15). For instance, several redox-sensitive transcriptional regulators exist in bacteria (16, 17) and these regulators usually control the expression of genes involved in defense against oxidative stress (18, 19). However, alteration of bacterial signaling by $H_2O_2$ can also be used by host cells as a defense mechanism. Intestinal cells produce low $H_2O_2$ concentrations that interferes with bacterial signaling and weakens the fitness of potential intestinal pathogens (20).

In this study, we addressed a significant open question in the field: concentrations of ROS that can be achieved *in vivo* are sublethal for S. aureus, yet NOX2-derived ROS play a crucial role in the host defense against this microorganism. We found a major impact of sublethal ROS concentrations on gene expression in *S. aureus*. The importance of understanding the interaction of ROS with this important pathogen is severalfold: first, it should provide at least a part of the answer to the oldest question in CGD research, namely why do patients that cannot generate ROS have such difficulties to defend themselves against *S. aureus*; second, a better knowledge of the effect of $H_2O_2$ on *S. aureus* transcriptome might provide new therapeutic targets within *S. aureus*, which is now considered as a high priority pathogen for the development of new treatments by the WHO.

# MATERIALS AND METHODS

## Bacterial Strains, Culture Medium and Growth Analysis

All bacterial strains used in this study are summarized in **Table 1**. All strains were cultured at 37°C in tryptic soy broth (TSB) or on tryptic soy agar (TSA). When antimicrobial agents were indicated, they were added to the following concentrations: erythromycin (Sigma-Aldrich) 5 μg $ml^{-1}$ and chloramphenicol (AppliChem) 10 μg $ml^{-1}$. For growth restoration of the *carA* mutant strain, the medium was supplemented with uracil 2.5 mM (Sigma-Aldrich).

Growth dynamics was followed on 384-wells plate (Corning #3640) using a plate reader Infinite 200 Pro (Tecan) at 37°C with an orbital shaking of 5 mm. The absorbance was measured every 6 minutes at optical density 595 nm ($OD_{595nm}$).

## *In Vitro* Exposure to $H_2O_2$

After an overnight culture, *S. aureus* was inoculated in TSB to an $OD_{595nm}$ of 0.01 and 50 μL of the bacterial culture were dispensed on a 384-wells plate (Corning #3640) and incubated

**TABLE 1** | Bacterial strains and plasmid.

| Strain or plasmid | Relevant genotype or characteristic(s) | Source/reference |
|---|---|---|
| *S. aureus* strains | | |
| JE2 | *S. aureus* USA300 LAC plasmids-cured | NTML |
| RN4220 | Restriction-defective strain which accepts foreign DNA | (21) |
| NE1526 | JE2-derived strain with a transposon in *carA* gene | NTML |
| NE1301 | JE2-derived strain with a transposon in *pyrB* gene | NTML |
| NE356 | JE2-derived strain with a transposon in *pyrE* gene | NTML |
| NE1759 | JE2-derived strain with a transposon in *pyrF* gene | NTML |
| NE1048 | JE2-derived strain with a transposon in *pyrP* gene | NTML |
| *E. coli strains* | | |
| DH5α | Routine laboratory strain | |
| BW25113 | F-Δ(araD-araB)567ΔlacZ4787(::rrnB-3)rph-1Δ(rhaD-rhaB)568hsdR514 | CGSC |
| JW0031-1 | BW25113 Δ*carB*745::*kan* | (22) |
| *Plasmid* | | |
| *pMK4* | *E. coli-S. aureus* shuttle plasmid, $Cam^r$ $Amp^r$ | (23) |

at 37°C with orbital shaking. When bacterial growth reached the early exponential phase ($OD_{595nm}$ ~0.2-0.3), $H_2O_2$ was added at different final concentrations (0 mM, 0.00061 mM, 0.00122 mM, 0.00244 mM, 0.00488 mM, 0.00977 mM, 0.01953 mM, 0.03906 mM, 0.07813 mM, 0.156 mM, 0.313 mM, 0.625 mM, 1.25 mM, 2.5 mM, 5 mM, 10 mM, 20 mM, 40 mM, 80 mM, 160 mM and 320 mM). $H_2O_2$ 30% (Sigma-Aldrich) was freshly diluted in TSB before use. Briefly, $H_2O_2$ was diluted at 11-fold desired concentration and 5 µL was added to the 50 µL bacterial culture (total volume per wells: 55 µL). A dose-response curve based on the growth rate during exponential phase curves after $H_2O_2$ exposure was made. We used the *Doubling Time Software v1.0.10* (http://www.doubling-time.com) to estimate the growth rate (24). It allowed us to identify the highest sublethal concentration, where ~30% of bacterial growth rate of the exponential phase was affected after addition of $H_2O_2$.

## RNA Isolation, RNA-Seq and qRT-PCR

Total RNA was extracted as described previously by Fischer et al. (25). Briefly, overnight cultures were diluted to an $OD_{595nm}$ of 0.01 and grown in fresh TSB for 3h at 37°C with constant shaking (180 rpm). Bacteria were washed and lysed with lysostaphin 25 µg/ml (Sigma) and RNA was isolated with RNeasy mini kit plus (Qiagen). gDNA was removed with DNase I digestion as previously described by Schuster et al. (26). Total RNA was quantified with Cubit or Nanodrop. RNA-Seq analysis was made as described by Mikheyeva et al. (27). Briefly, the RNA integrity was determined using Agilent 2100 BioAnalyzer (Agilent Technologies) to verify the quality of extracted RNA and one microgram of total RNA was ribo-depleted with the Ribo-Zero kit (Illumina). For the library preparation, the truseq total RNA stranded was used. Using Illumina HiSeq 4000 sequencer, oriented 50 bases single-read sequencing was performed. Finally, RNA-Seq and data analysis were carried as described in Cherkaoui et al. (28). Low quality reads and reads containing adapter and poly-N were removed and remaining reads were aligned on USA300 genome (accession number: CP000255). For qRT-PCR, total RNA was reverse transcribed using PrimerScript Reverse Transcriptase (TaKaRa) and genes were quantified using a Brilliant SYBR green master mix (Agilent). The primers used in this study are described in **Table 2**. For RNA isolated after phagocytosis, a preamplification with the TaqMan™ PreAMp Master Mix Kit (Applied Biosystems) was performed. Quantitative PCR (qPCR) reactions were performed in a Bio-Rad CFX96 and normalized using intensity levels recorded for the *hu* gene as previously described in Garzoni et al. (29).

## Construction of Plasmid With Constitutive *carA* Expression

A polymerase chain reaction (PCR) amplification of *carA* gene was made using reverse and forward primers and JE2 chromosomal DNA as template. Forward primer contained a KpnI tail and reverse primer a Pst1 tail. A *Pfu* DNA polymerase (Promega) was used for PCR products amplification. Fragments were ligated using KpnI and Pst1 enzymes in a custom designed pmk4-based vector containing a P*glyS* promoter. The resulting plasmid was electroporated into the non-restrictive *S. aureus* strains RN4220 prior to transfer to *carA* mutant strains. Complemented strains were selected with chloramphenicol 10 µg/ml. The construction was sequenced and verified. Restoration of the *carA* function was confirmed by growth kinetic and by PCR of *carA* expression.

## EdU Click Labeling of Newly Replicated DNA in *S. aureus*

We monitored DNA synthesis as described in Martel et al. (30). Briefly, overnight cultures of *S.aureus* were diluted to $OD_{595nm}$ of 0.01 in 30 ml TSB and grown at 37°C with agitation until $OD_{595nm}$ of 0.2 was reached. The culture was separated in 12 samples of 2ml. EdU from Click-iT EdU® assay (Invitrogen) was added (final concentration 0.12 mM) and incubated for 15 minutes at 37°C. A control group was left untreated to assess the unspecific coloration of the Alexa Fluor®-azide. $H_2O_2$ was added at a final concentration of 20 mM to the $H_2O_2$ treated group. Bacteria were grown for an additional 30 minutes at 37°C with shaking and growth was stopped by fixation with 90% methanol. Fixed bacteria were washed with 1.5 ml PBS and permeabilized with 200 µL lysostaphin (80 µg/mL in TE buffer)

**TABLE 2** | Primers.

| Primers (5' - 3') Gene | Forward | Reverse |
|---|---|---|
| ***HU*** | CCTCAAAGTTACCGAAACCAA | AGCTGGTTCAGCAGTAGATGC |
| ***carA*** | TTCCAGGGATTGCAGGTGTT | AACGCCATCTGGAGCCATTG |
| ***carB*** | CAGCCACAAGGGAAAACAGC | TTCCAGGGATTGCAGGTGTT |
| ***pyrB*** | TCTGGTGAACGTCAACTACCA | TACCATCACCAGCATTCGCA |
| ***pyrC*** | CGCGCAGGCATTCATGTTAC | GTGCATGGTCTGTTGCGATAC |
| ***pyrD*** | AAACACAACGCGACAACGAG | AACGTGCGATGCCTTTGTTC |
| ***pyrE*** | CCTTTAGTTCGAGGCGCAATC | GTGACTGAAGATCCCCCTGTC |
| ***pyrF*** | GCTGCTGGTGGCGTAAAAAT | TATGCGTCGAACCAAGCTGT |
| ***pyrG*** | TTGGCGGTACAACAGGTGAT | ATGTTGCGTTGGCTTCGTTT |
| ***pyrP*** | AGCAGCGTTACTAGCTTCGG | TCCCGTGATAATTGGCGTGA |
| ***pyrR*** | TGCCGCAATACAACGTACAG | CCGTTCGACCAGTATACAGCA |
| **Cloning** | | |
| | **Forward** | **Reverse** |
| ***carA*** | GGCGGGTACCAAGGAGGAACAAT CATGCAAAGCAAACGTTATCTAGTG | CGATCTGCAGTTAGGCATTGATATGACGCTCC |

and incubated 15 minutes at 37°C as described in Rodriguez and Kuehn (31). EdU incorporation was revealed by the adjunction of 200 µl of Click-iT® reaction cocktail with Alexa Fluor488®-azide prepared according to the manufacturer's instructions. After 30 minutes of incubation at room temperature, bacteria were washed with PBS and resuspended in 1.5ml of filtered PBS with 1% human serum albumin. Five µl of 200µl/ml DAPI (Applichem) was added to the samples and incubated at room temperature for 10 minutes. Samples were visualized by a Gallios Flow Cytometer (Beckman Coulter) set for 488 nm (laser) with a 525/40 filter with 30'000 events per samples. Data were analyzed with Kaluza Analysis Software (Beckman Coulter). DAPI-negative events were not considered as bacterial cells and excluded. The percentage of cells with incorporated EdU and the arithmetic mean of fluorescence of EdU-positive cells were reported to the percentage of corresponding events in the untreated condition to compare the different experiments.

## Isolation of Human Neutrophils

Human neutrophils were purified from 10 ml citrated blood samples of healthy donors after obtaining their informed consent. Total blood cells were separated by sequential Ficoll-Hypaque differential density centrifugation as described in Genestet et al. (32). Neutrophils in the pellet were resuspended in phosphate-buffered saline (PBS) solution. Erythrocytes were lysed twice with a frozen hypotonic lysis buffer as described by Dri et al. (33). Briefly, the lysis buffer contained 155 mM $NH_4Cl$, 10 mM $KHCO_3$, 0.1 mM EDTA for a pH of 7.4. After erythrocytic lysis, cells were centrifugated for 10 minutes at 460 g at 4°C and neutrophils were resuspended in $Ca^{+2}$ and $Mg^{+2}$ free HEPES-buffered saline (HBS) solution containing 140 mM NaCl, 5 mM glucose, 5 mM KCl, 5 mM HEPES and 0.2% of bovine serum albumin (BSA). Cells were kept on ice until use. Directly before use, cell suspensions were supplemented with 1 mM $CaCl_2$ and 1 mM of $MgCl_2$.

## Microbicidal Activity Assay With Human Neutrophils

The microbicidal activity of neutrophils was assessed according to the method described in Decleva et al. (34). In brief, $4x10^6$ neutrophils/ml were incubated at 37°C with shaking 160 rpm with serum-opsonized *S. aureus* at a bacteria/neutrophil ratio of 3:1. After one hour of incubation, aliquots were diluted 50 times in water with NaOH (pH 11) for 5 minutes in order to lyse neutrophils. Then, tubes were vortex and diluted into 0.9% NaCl solution and plated on Petri dishes. The next day, CFU were counted and the percentage of killing was calculated according to the number of CFU at T0.

## Statistics

GraphPad Prism 8 for Windows (GraphPad Software, San Diego, USA) and Rstudio 1.2.5001 (Rstudio, Boston, USA) were used for data processing, graph plotting and statistical analysis. Pairwise comparisons were performed to detect differentially expressed transcripts between $H_2O_2$ treated and control condition and statistical significance (False Discovery rate < 0.05) was determined using DeSeq2 3.10 (available at http://www.bioconductor.org/packages/release/bioc/html/DESeq2.html). For the $IC_{50}$ analysis and curve fit, a normalized log(response) inhibition model was used. Where Y = growth rate (percentage), X = $H_2O_2$ concentration, $IC_{50}$ = $H_2O_2$ concentration that decrease growth rate from 50%. Kruskal-Wallis test with Dunn's multiple comparison tests were applied to compare the EdU labeling incorporation in newly replicated DNA.

# RESULTS

## Hydrogen Peroxide Significantly Affects the Expression of Genes Involved in Pyrimidine Metabolism

We first investigated growth of *S. aureus* JE2 strain at different $H_2O_2$ concentrations (**Figure 1**). $H_2O_2$ was added at the early exponential phase ($OD_{595nm}$ 0.25) and the effect on growth

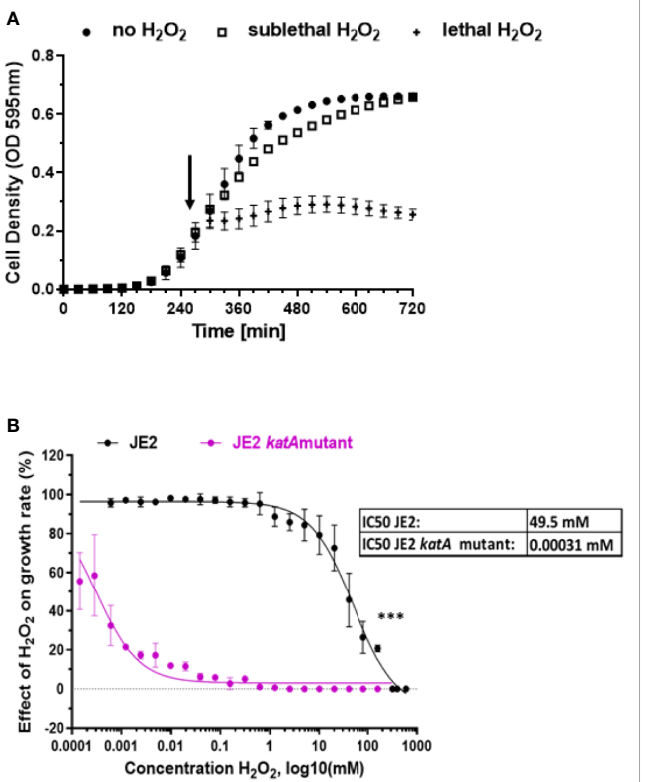


**FIGURE 1** | Effect of $H_2O_2$ on *S. aureus* growth. **(A)** *S. aureus* wild-type JE2 strain growth in absence of $H_2O_2$ (●) and in the presence of two $H_2O_2$ selected concentrations: sublethal (20mM) (□) or lethal (160 mM) (+). $H_2O_2$ was added in the early exponential phase, as indicated by an arrow. **(B)** Effect of 22 different concentrations of $H_2O_2$ on wild-type *S. aureus* JE2 strain and its corresponding *katA* mutant and compared to growth in the absence of $H_2O_2$. Normalized values were expressed as percent of growth in the absence of $H_2O_2$ on the same experiment. Results represent the mean ± SD of three independent experiments (four replicates for each $H_2O_2$ concentration). A nonlinear regression model was applied to estimate the $IC_{50}$. ***$p < 0.001$.

dynamic was observed during the entire exponential phase (**Figure 1A**). We tested 22 different concentrations of $H_2O_2$, ranging from 0.61 µM to 320 mM and we calculated the growth rate during the exponential phase after addition of the respective amounts of $H_2O_2$. Based on these results, the apparent $IC_{50}$ of $H_2O_2$ for JE2 growth was 49.5 mM (**Figure 1B**). 20 mM $H_2O_2$ is the highest concentration which did not affect growth in a significant statistical manner (p=0.053). Thus, we defined 20 mM $H_2O_2$ as the highest sublethal concentration affecting moderately bacterial growth. This concentration was used for most experiments shown below. In order to verify that the growth of *S. aureus* is indeed affected by $H_2O_2$, we tested similar $H_2O_2$ concentrations on a catalase-deficient strain (JE2 *katA* mutant). Catalase is an enzyme that metabolizes $H_2O_2$ into $O_2$ and $H_2O$. In the absence of catalase expression, *S. aureus* is approximatively $10^7$ times more susceptible to $H_2O_2$ killing with an estimated $IC_{50}$ of 0.31 µM (**Figure 1B**).

The impact of sublethal $H_2O_2$ concentration on gene expression in *S. aureus* was addressed by RNA-Seq. $H_2O_2$ was added at the early exponential phase and after one hour of exposure to 20 mM (or 0 mM for control conditions), total bacterial RNA was extracted and their respective transcriptome was determined. This time point was chosen in order to study bacterial response after the immediate stress response such as catalase expression. **Figure 2** illustrates the differential expression analysis for *S. aureus* treated with $H_2O_2$ *versus* untreated. We observed 190 differentially expressed genes with a fold change ≤ - 2 and ≥ 2 with statistical significance (False Discovery Rate (FDR)). Among these genes, 98 were upregulated and 92 downregulated (**Table 1** and **Supplementary Data**). Genes with unknown function (n=85, 44.7%) were removed from **Table 1**. In order to identify gene categories affected by the presence of $H_2O_2$, we analyzed RNA-seq data based on TIGRFAM gene categories. **Figure 2A** illustrates the number of differentially expressed genes on the total number of genes in the corresponding TIGRFAM main role. TIGRFAM is a protein families database designed to support genome annotation (35, 36). Only genes with expression that were statistically (FDR<0.05) and significantly changed (fold change ≤-2 or ≥2) were analyzed. Pathways including DNA replication, recombination and repair and DNA interactions had the highest number of genes upregulated, followed by riboflavin, FMN and FAD pathways. The upregulated genes belonged to various families, including genes involved in riboflavin metabolism [typically involved in redox response (37)], phage genes, genes involved in DNA repair, as well as several gene encoding non-identified hypothetical proteins. The downregulated genes belonged to different gene families, including degradation of proteins, peptides, and glycopeptides, ribosomal proteins or toxin production and resistance. Interestingly, the category of genes involved in pyrimidine ribonucleotide biosynthesis was the pathway with the most important downregulation. There was an almost complete downregulation of the different genes involved in the pyrimidine ribonucleotide biosynthesis (fold changes ranging from -7 to -2) (**Figure 2B**). We confirmed a dose-dependent downregulation observed in the RNA-Seq by qRT-PCR (**Figure 2C**). We decided to focus our research on this gene family.

## Inactivation of *carA* and *pyrP* Genes Affects *S. aureus* Growth

Next, we addressed the impact of inactivation of *pyr* genes on the fitness of *S. aureus*. To study this question, we used a library of mutant strains, the Nebraska Transposon Mutant Library (NTML) (38, 39), to address the question of inhibition of the pyrimidine pathway. We used six mutants of the pyrimidine biosynthesis pathway: *carA*, *pyrB*, *pyrD*, *pyrE*, *pyrF*, *pyrP*. The biosynthesis of pyrimidine is divided into two different pathways: i) the *de novo* pathway, which uses glutamine, ATP and bicarbonate for uridine monophosphate (UMP) synthesis and, ii) the salvage pathway, which uses extracellular uracil to synthetize UMP (**Figure 3A**). Several *pyr* genes such as pyrP, *pyrB*, *pyrC*, *carA*, *pyrF* and *pyrE* are located on the same operon and transcribed from a single promoter (**Figure 3B**).

We first investigated the growth of *pyr* mutants and we observed two mutants displaying a growth defect. Growth was strongly affected for *carA* and *pyrP* mutants (**Figures 4A, B**). The growth of *pyrB*, *pyrD*, *pyrE*, *pyrF* mutants was not affected (**Figures 1A–D** and **Supplementary Data**).

To analyze the effect of $H_2O_2$ on each *pyr* mutant, we exposed each mutant to eight different $H_2O_2$ concentrations, ranging from 2.5 mM to 160 mM of $H_2O_2$ for one hour and observed the effect on their growth rate. The growth rate was normalized according to the growth rate in absence of $H_2O_2$ and compared with the parental strain. The estimated $IC_{50}$ for each mutant is represented on each graph. *pyrB*, *pyrD*, p*yrE* and *pyrF* mutants have a $H_2O_2$ sensitivity close to the parental strain with an $IC_{50}$ respectively at 80.5 mM, 73.5 mM, 72.7 mM and 46.7 mM respectively (**Figure 2** and **Supplementary Data**). In contrast, *pyrP* and *carA* mutants were more sensitive to $H_2O_2$ than the parental strain with $IC_{50}$ estimated to 14.5 mM and 11.4 mM (**Figures 5A, B**).

Among all *pyr* mutants found in the NTML, we observed that loss of function of two *pyr* genes, *carA* and *pyrP*, induced a drastic alteration of bacterial fitness. The *carA* gene encodes for the small subunit of the carbamoyl phosphate synthase, a subunit of a large enzymatic complex associated with CarB, initiating the first reaction in pyrimidine and arginine metabolism (40) (**Figure 3A**). The *pyrP* gene encodes for an uracil permease located in the bacterial membrane, which transports uracil into the cell (41) (**Figure 3A**). Given the strong phenotype of the *carA* mutant, showing important growth defect and an increased sensitivity to $H_2O_2$ in *S. aureus*, we focused our investigations on this strain.

## *carA* Deletion Affects Growth and Sensitivity to $H_2O_2$

To demonstrate that *carA* gene mutation is involved in growth and sensitivity to $H_2O_2$, we first complemented the *carA* mutant with a pMK4 multicopy plasmid carrying *carA* gene under the control of the PglyS constitutive promoter (42, 43) (see materials

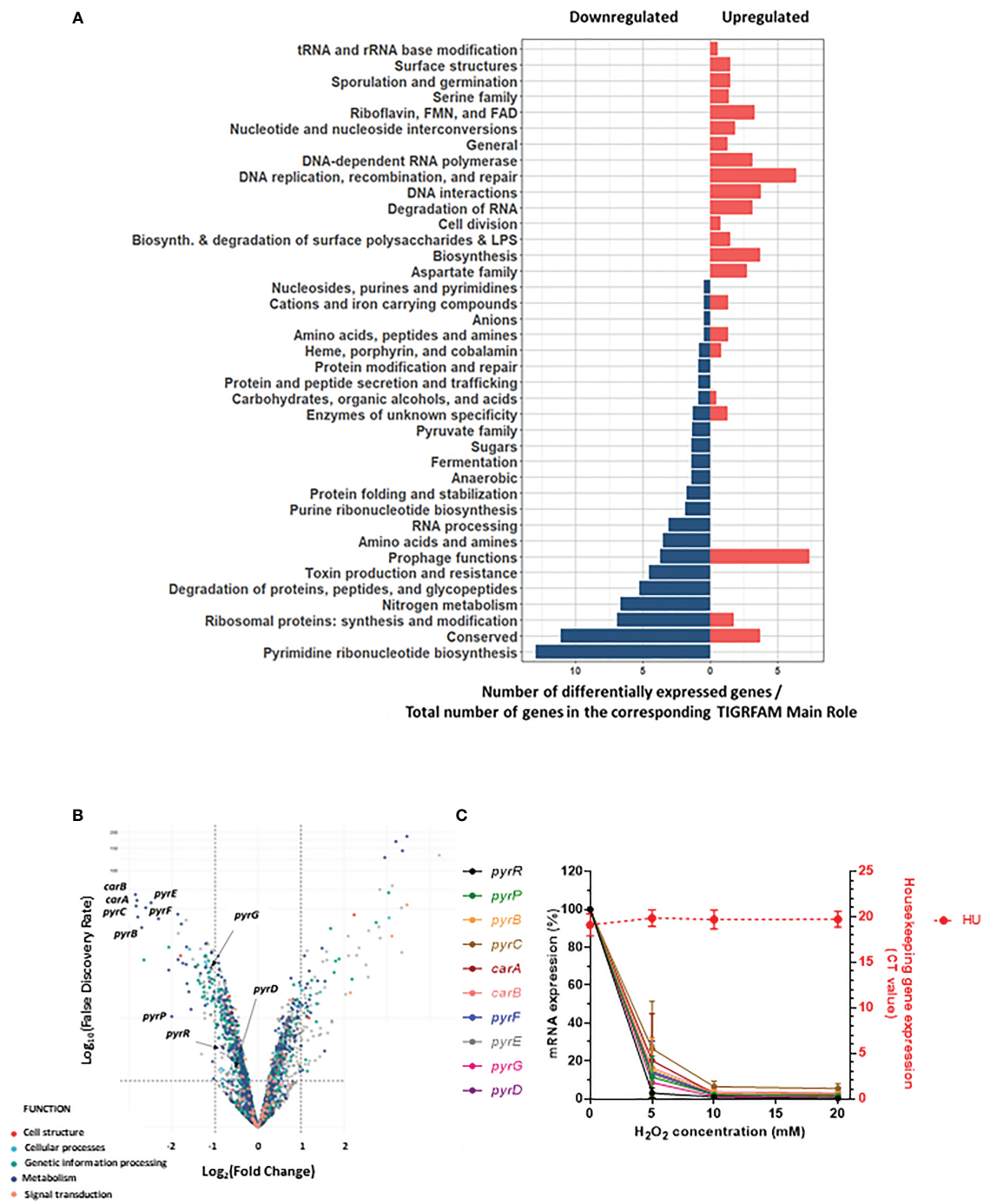


**FIGURE 2** | Downregulation of genes involved in pyrimidine metabolism after $H_2O_2$ exposure. **(A)** Pyramid plot displaying significant affected *S. aureus* genes in the presence of $H_2O_2$. From the total number of genes present in the TIGRAM main role category, the significant affected gene categories were selected, based on TIGRFAM category "Sub-Role" (FDR <0.05 and fold change ≤-2 or ≥2). Gene categories upregulated or downregulated upon $H_2O_2$ exposure are indicated in red or blue, respectively. **(B)** Volcano plot of RNA-Seq data representing the differential gene expression analysis of *S. aureus* JE2 exposed or not to one hour of sublethal $H_2O_2$. The upper left panel represents genes significantly (FDR <0.05 and Fold change ≤-2) downregulated after $H_2O_2$ exposure. The upper right panel represents genes significantly (FDR <0.05 and Fold change ≥2) upregulated after $H_2O_2$ exposure. RNA-Seq results were normalized using DESeq2 software. **(C)** Effect of different concentrations of $H_2O_2$ on *pyr* genes expression. Bacteria were exposed for one hour to different concentrations of $H_2O_2$. *pyr* gene expression was assessed by qRT-PCR. Relative expression levels were determined by comparing cycle threshold (CT) of each gene to the CT value of the *hu* gene for the same cDNA preparation. Left y axe represents mRNA expression of *pyr* genes and right y axe shows the minimal variation of *hu* CT values with different $H_2O_2$ concentrations (CT 20 +/- 0.3). These results represent the mean ± SD of three independent experiments (three replicates for each $H_2O_2$ concentration and three replicates for each gene).

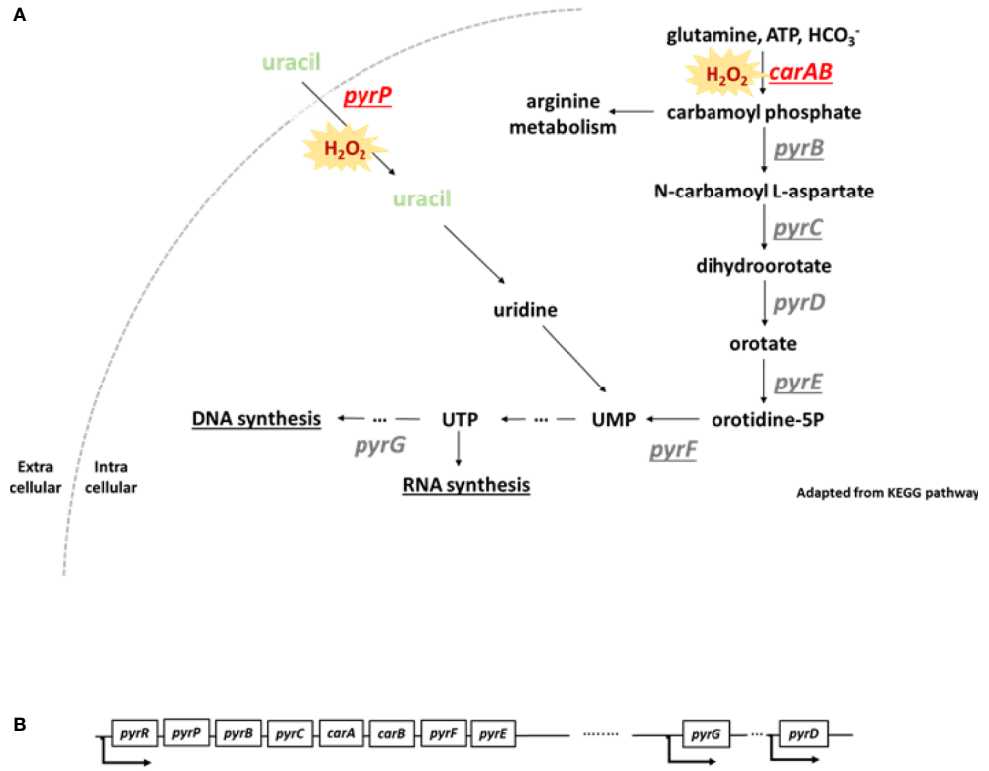


**FIGURE 3** | *pyr* operon and scheme of pyrimidine biosynthesis pathway. **(A)** Under oxidative stress, genes involved in pyrimidine biosynthesis are downregulated. Loss of function of *carA* and *pyrP* leads to an alteration of bacterial fitness. Pyrimidine metabolism is divided in two different pathways: the *de novo* and the salvage pathways. The *de novo* pathway uses glutamine, ATP and bicarbonate ($HCO_3^-$) in the cell to produce uridine monophosphate (UMP). The salvage pathway uses extracellular uracil to generate UMP. Loss of function of *carA* and *pyrP* genes leads to an alteration of bacterial fitness and a higher sensitivity to $H_2O_2$. UMP, uridine monophosphate, UTP, uridine triphosphate. The underlined genes are located on the same operon. This scheme was adapted from KEGG pathway. **(B)** Some of the *pyr* genes are located on an operon and transcribed from a single promoter. *pyrG* and *pyrD* are located further in the genome and transcribed with their own promoter.

and methods). Bacterial growth and sensitivity to $H_2O_2$ was further analyzed. CarA complementation restores *carA* mutant growth, similar to wild-type JE2 strain (**Figure 4B**). Moreover, the complemented strain showed a strong decreased sensitivity to $H_2O_2$ with an $IC_{50}$ of 107.6 mM, compared to the $IC_{50}$ of the *carA* mutant ($IC_{50}$ = 11.42 mM) (**Figure 5B**). These results show that loss of function of *carA* gene not only affects bacterial growth but also plays a role in the sensitivity to $H_2O_2$.

## Uracil Restores Growth Defect in *carA* Mutant

As uracil is a common and natural pyrimidine derivative, we supplemented the culture medium with uracil. As depicted in **Figure 4C**, 2.5 mM uracil led to a substantial recovery of growth of the *carA* mutant. We tested higher concentrations of uracil, but they did not further enhance bacterial growth and even had a deleterious effect on growth above 5 mM (data not shown). Note that the recovery was delayed and incomplete suggesting that this compensatory mechanism might be saturated and does not fully compensate for the loss of the *carA* gene. *carA* is also involved in arginine metabolism. We therefore tested different concentrations of arginine and did not observe any growth restoration (**Figure 4D**).

## $H_2O_2$ Induces a Decreased DNA Replication in *S. aureus*

We then wanted to study if the downregulation in pyrimidine metabolism observed after addition of sublethal concentration of $H_2O_2$ was due to a decreasedbacterial DNA replication. We performed an EdU-Click labeling assay and compared the incorporation of the nucleotide analog 5-ethynyl-2'-deoxyuridine (EdU) in *S. aureus* JE2 with and without 20 mM of $H_2O_2$ by flow-cytometry (**Figure 6**). $H_2O_2$ was added after EdU to avoid oxidation. EdU untreated *S. aureus* showed some unspecific binding of the Alexa Fluor488®-azide. In the absence of $H_2O_2$, EdU was actively incorporated in DNA over time while in the $H_2O_2$ treated group, we did not observe EdU incorporation after $H_2O_2$ treatment (**Figure 6A**). The percentage and the arithmetic mean fluorescence of EdU positive *S. aureus* were normalized to the EdU $H_2O_2$ untreated condition. We observed that, in presence of 20 mM of $H_2O_2$, DNA replication was limited while incorporation of EdU was increased by approximately 40% in the absence of $H_2O_2$ (**Figures 6B, C**). These results suggest that the effect $H_2O_2$ on genes involved in pyrimidine metabolism leads to a decreased DNA replication.

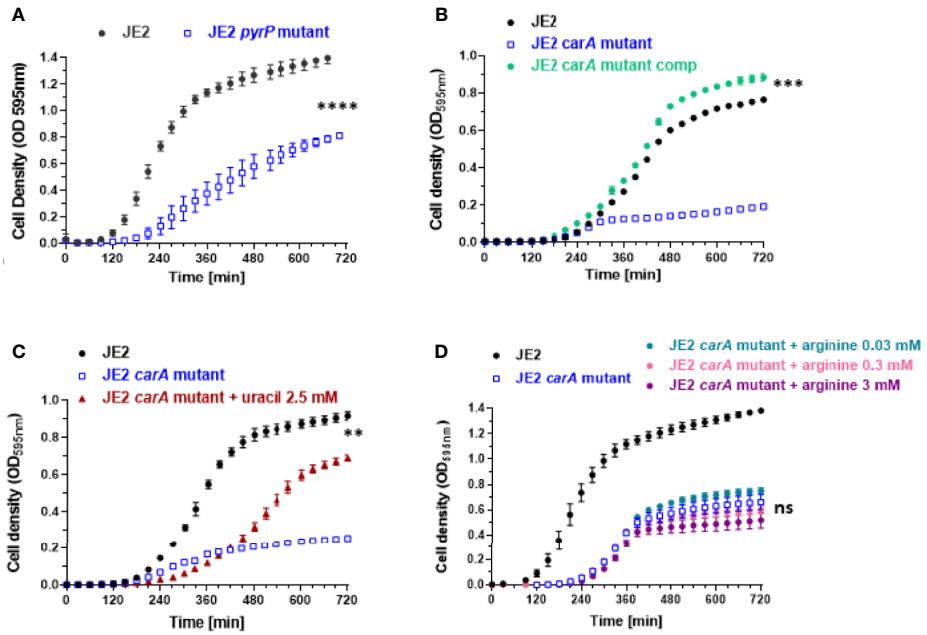


**FIGURE 4** | Effect of *carA* and *pyrP* mutations on bacterial growth and partial rescue of *carA* mutant with uracil and *carA* complementation. **(A, B)** Bacterial growth analysis of wild-type *S. aureus* JE2 strain, *pyrP* and *carA* mutants (from the Nebraska transposon mutant library) and *carA* mutant carrying a multicopy plasmid expressing *carA* (JE2 *carA* mutant comp). **(C)** Partial growth restoration of *carA* mutants after supplementation of 2.5 mM of uracil in the medium. **(D)** Absence of growth restoration with different arginine concentrations. Results represent the mean ± SD of three independent experiments (four replicates for each strain). **p < 0.005, ***p < 0.001, ****p < 0.0001, ns p > 0.005 by Mann-Whitney test.

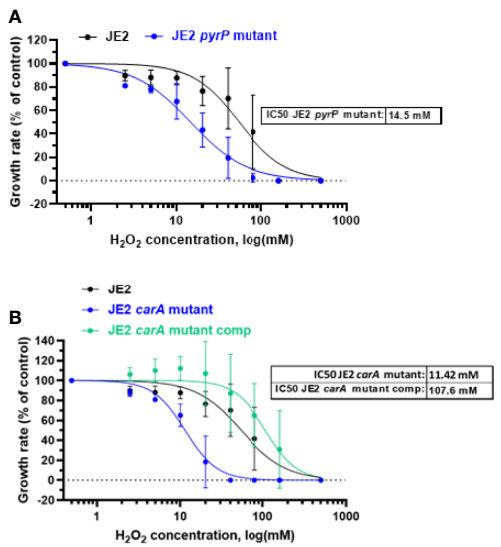


**FIGURE 5** | $H_2O_2$ effect on bacterial growth rate. Growth of wild-type *S. aureus* JE2 strains and *pyrP* **(A)** and *carA* mutants and *carA* mutant carrying a multicopy plasmid expressing *carA* (JE2 *carA* mutant comp) **(B)** was analysed in the presence of 8 different concentrations of $H_2O_2$ and compared to growth in the absence of $H_2O_2$. Normalized values were expressed as percent of growth in the absence of $H_2O_2$ on the same experiment. Results represent the mean ± SD of two independent experiments (four replicates for each $H_2O_2$ concentration). A nonlinear regression model was made to estimate the $IC_{50}$. p > 0.005.

## *carA* Gene Is Not Involved in Survival After Neutrophil Phagocytosis

Due to the increased sensitivity of *carA* mutant to $H_2O_2$, we investigated the impact of the lack of *carA* expression in a more complex model than *in vitro* $H_2O_2$ exposure. We first analyzed the expression of *carA* gene after one hour of phagocytosis (**Figure 7A**) and observed downregulation similarly to what we observed *in vitro*. Then, we assessed the survival of JE2, *carA* mutant and *carA* complemented strains after phagocytosis by human neutrophils. Opsonized *S. aureus* strains were incubated with freshly isolated human neutrophils for 1 hour and their survival was measured by counting the colonies on agar plate after 24 hours, in presence or absence of an irreversible NOX2-inhibitor (DPI). The number of CFUs was normalized according the CFUs number at T0. We observed that in presence of neutrophils, the survival rate dropped around 40% for all the three strains (**Figure 7B**). The role of the ROS generating NOX2 was confirmed by the fact that the killing activity of neutrophils was inhibited by 5 µM of DPI. Therefore, the absence of *carA* expression did not affect the bacterial survival after phagocytosis in presence and in absence of ROS.

## DISCUSSION

The aim of this study was to understand why ROS are crucial in the host defense against *S. aureus*. We observed important changes in gene expression at sublethal $H_2O_2$ concentrations,

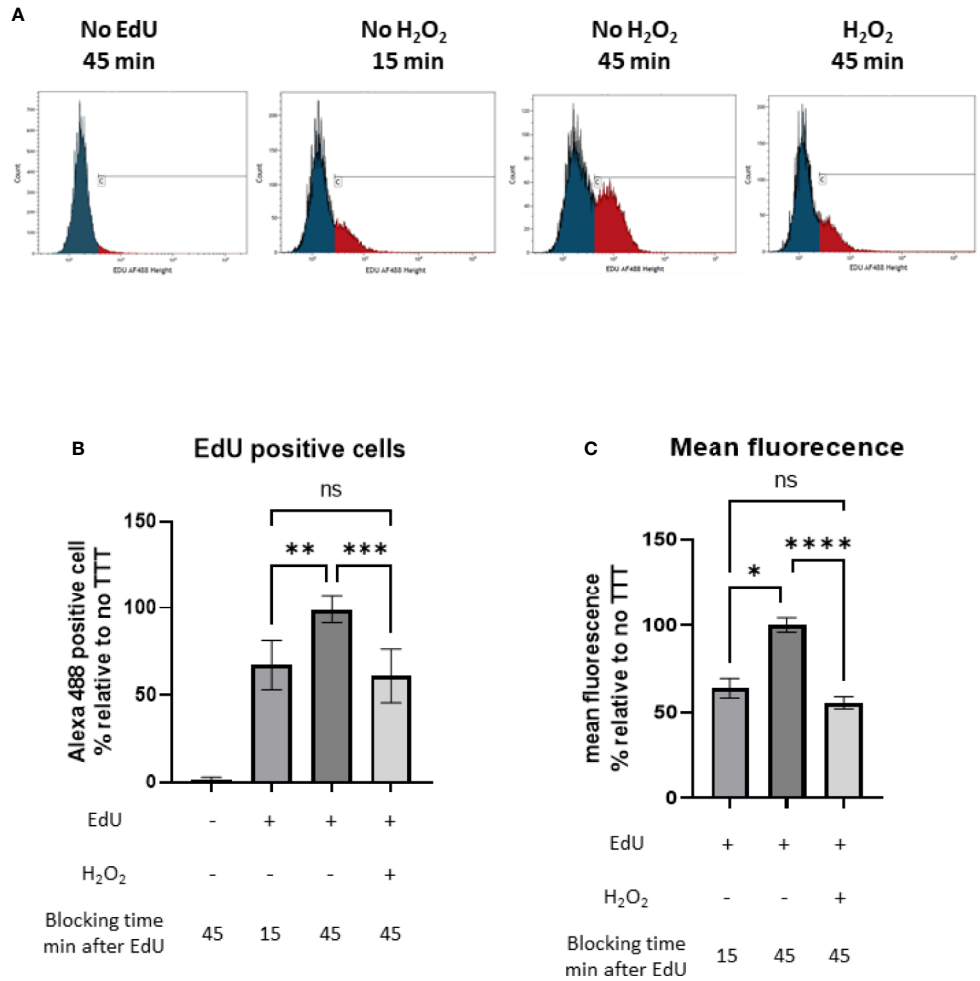


**FIGURE 6** | Monitoring of the DNA synthesis by following the incorporation of the nucleotide analog EdU (5-ethynyl-2′-deoxyuridine), Click-iT® assay (Invitrogen) with and without $H_2O_2$ treatment. **(A)** Number of events under fluoresence intensity. Blue and red peaks represent respectively unspecific binding of Alexa Fluor488®-azide (defined by the control without EdU) and bacteria with active DNA replication. **(B)** Percentage of EdU positive *S. aureus* normalized to the untreated 45 min condition. **(C)** Arithmetic mean fluorescence of EdU positive *S. aureus* normalized to the untreated 45 min condition. These results represent mean ± SD of three different experiments (three replicates for each condition). ns, not significant, *p < 0.05, ** p < 0.005, ***p < 0.001, ****p < 0.0001 by Kruskal-Wallis test with Dunn's multiple comparison tests.

in particular a concerted downregulation of enzymes of pyrimidine metabolism. This downregulation was caused by a decreased in DNA replication and was strongly associated with decreased extracellular, but not intracellular survival of the bacteria. We hypothesize that this is a long-term survival mechanism of *S. aureus*: avoiding growth of extracellular *S. aureus* which can cause severe infection and lead to death of its host, while assuring its intracellular survival.

After one hour of exposure to a sublethal concentration of $H_2O_2$, the number of upregulated and downregulated genes was approximately equal, suggesting that under our experimental conditions, $H_2O_2$ does not lead to unspecific toxicity, but rather induces a cellular response. Interestingly among upregulated genes, the expression of genes involved in antioxidant response was unchanged, most likely because they are expressed within minutes after the onset of the oxidative stress and already decreased to normal levels one hour after addition of $H_2O_2$. Our hypothesis was that $H_2O_2$ affects *S. aureus* general fitness and we decided to focus on downregulated genes. Among downregulated genes, we observed that most of the genes involved in pyrimidine biosynthesis were strongly affected in *S. aureus* following one hour of exposure to a sublethal concentration of $H_2O_2$. We identified two genes in this family, *carA* and *pyrP*, that were important for bacterial growth in rich medium and $H_2O_2$ sensitivity. We observed impaired DNA replication after $H_2O_2$ exposure. This decrease in DNA replication and transcription was dependent on the downregulation of pyrimidine biosynthesis. This concerted response represents a compensation mechanism due to the presence of $H_2O_2$ ensuring bacterial survival. Indeed, similar

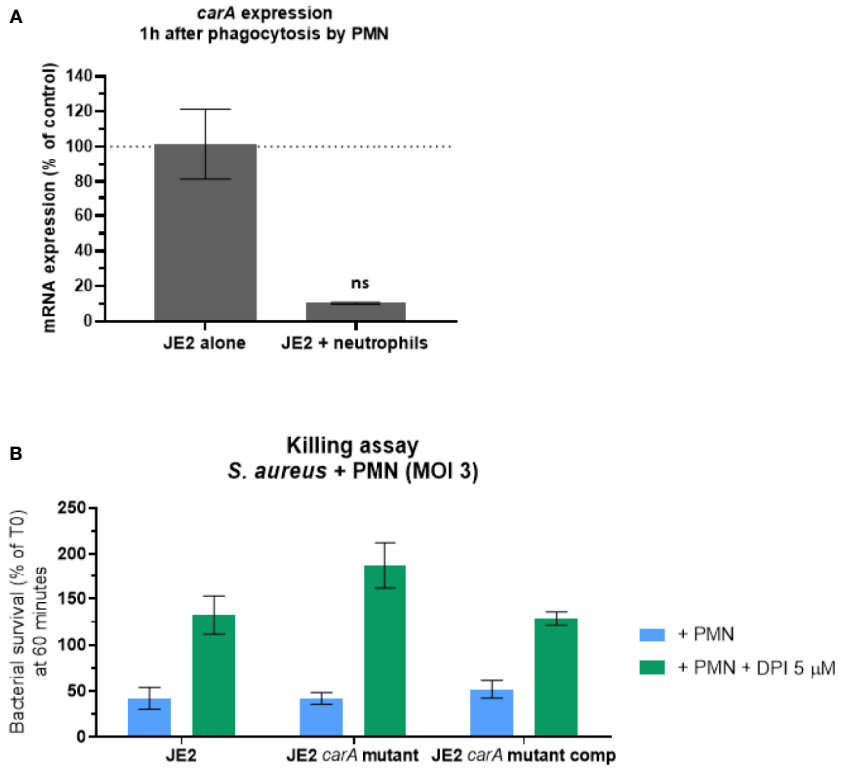


**FIGURE 7** | *carA* expression and *S. aureus* survival 60 minutes after phagocytosis by human neutrophils. **(A)** Expression analysis of *carA* with qPCR one hour after phagocytosis by human neutrophils. Gene expression was normalized with the gene expression in non-phagocytosed bacteria. These results represent the mean ± SD of two independent experiments. **(B)** Bacterial survival was estimated after 60 minutes of co-culture with human neutrophils with a multiplicity of infection (MOI) of 3. ROS production inside the phagosome was either conserved (blue) or inhibited (green) by 5 µM of diphenyleneiodonium (DPI). CFU counts following the experiment were normalized according CFU count from the initial inoculum. These results represent the mean ± SD of three independent experiments. ns, not significant, p>0.05 by Kruskal-Wallis test.

amount of $H_2O_2$ resulted in bacterial death of a *carA* mutant, a non-compensable mutation. We then confirmed that the expression of *carA* was also decreased after one hour of phagocytosis by human neutrophils. Furthermore, the fitness of the *carA* mutant was particularly affected and could be reversed by genetic rescue. Yet, survival of *carA* mutant after phagocytosis by human neutrophils was not different from the parental strain.

Pyrimidine metabolism leads to the formation of pyrimidine nucleotides (namely uracil, cytosine and thymine), which are used for nucleic acid synthesis (44), energy production (UTP or CTP) and other key cellular functions (45). Both *carA* and *pyrP* are involved in the first step of two pathways, *de novo* and salvage pathways, and, unlike the other genes in the pathway, they are crucial, and no alternative pathway can substitute their function. We think that in nutrient rich media, the salvage pathway is working, as bacteria are able to grow, however it is less efficient than the *de novo* synthesis pathway, explaining why the growth of the *carA* mutant is delayed (but not inhibited as can be seen in minimal media).

It has been observed that *carA* plays an important role in virulence in other bacterial species, such as *Pseudomonas syringae* (46), *Escherichia coli* (47), *Xanthomonas citri* (48) and *Francisella tularensis* (49). Similar to the genomic organization characterized in *B. subtilis*, several *pyr* genes (*pyR*, *pyrP*, *pyrB*, *pyrC*, *carA*, *carB*, *pyrF* and *pyrE*) of *S. aureus* are located on an operon and transcribed from a single promoter (**Figure 3B**). The transcription of the operon is negatively regulated by the binding of PyrR to specific anti-termination sites [described in detail in Turnbough and Switzer (50)]. PyrR peptide sequence does not contain cysteines redox-sensitive residues (44). However, two known redox-sensitive transcription factors, MgrA and SarZ, are known to negatively regulate expression of pyrimidine genes under oxidative stress (9, 51). Both regulators have a direct effect on *pyrR* but MgrA also controls the expression of genes involved in biosynthesis of another *pyr* gene that are not located on *pyr* operon (51), such a *pyrG*, which was also downregulated in our transcriptomic analysis. This finding supports a potential role of MgrA through an additional transcription factor. The specific redox regulation of MgrA and SarZ effect on the downregulation of genes involved in pyrimidine biosynthesis pathway could be specifically addressed using redox biochemical approach, such as the biotin switch assay (52).

Nucleotide clumping of the bacterial genome has been described as another type of bacterial response to oxidative

stress (53). This response is regulated by MgrA (53) and participates to bacterial stress tolerance by protecting the genome against ROS. Interestingly, the effect of $H_2O_2$ on genes involved in pyrimidine biosynthesis pathway is not specific to *S. aureus* and a study in *E. coli* demonstrated that $H_2O_2$ also induces a downregulation in genes involved in nucleotides and ribonucleotides production process (54).

Intriguingly, we observed a downregulation in *carA* expression following intraphagosomal oxidative stress, but we did not observe any difference in the survival of *carA* mutant and the parental strain, in the presence or absence of intraphagosomal ROS. Two aspects of this difference are relevant for this discussion: i) the underlying biochemical mechanism, and ii) the impact on the *S. aureus* reservoir for long-term survival as observed in numerous chronic infections.

A possible biochemical explanation for the decreased expression of *carA* in intracellular survival would be a high level of pyrimidine or pyrimidine precursors, in particular uracil, within the phagosome. To the best of our knowledge, phagosomal concentrations of the respective metabolites have not been measured. Another explanation would be changes in metabolism of intracellular bacteria leading to a decreased catabolism of pyrimidine or pyrimidine precursors. A third aspect that might contribute to the decreased importance of *carA* for phagosomal survival might be the decreased growth rate of intracellular *S. aureus* leading a decreased requirement of nucleotide precursors. This type of behavior has been observed during prolonged residency of *S. aureus* in non-phagocytic cells (55).

In summary, we demonstrated a novel and selective mechanism allowing *S. aureus* to survive in the presence of ROS, namely a coordinated downregulation of the pyrimidine biosynthesis pathway and a decreased in DNA replication. This downregulation has the interesting feature to selectively interfere with the growth of extracellular, but not intracellular *S. aureus*. Given this dichotomy, our results suggest that *S. aureus* is not a naive victim of host defenses in this situation but has developed an evolutionary survival strategy by modulating growth rate. Future studies should investigate the mechanisms described here in clinical strains. In our study where RNA-Seq data needs to be interpreted in the context of the entire genome, the choice of the JE2 strain was pertinent and allowed to uncover this novel *S. aureus* survival strategy. The importance of this "go unnoticed" strategy may contribute to the high mortality of CGD patients following *S. aureus* infection [at least if untreated (7)] and contribute to their long-term epidemiological success in the numerous people carrying *S. aureus*.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/ena, PRJEB43496.

## ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

HB, AL, VJ, PF, and KK designed experiments. HB, MR, AR, EB, FL, MM, and MS performed experiments. HB, VJ, FL, PF, and KK interpreted the results. NG performed bioinformatics analysis. HB and VJ wrote the manuscript. VJ, MS, JS, PF, and KK revised the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

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REVIEW
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# Reactive Oxygen Species in Macrophages: Sources and Targets

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Reactive oxygen species (ROS) are fundamental for macrophages to eliminate invasive microorganisms. However, as observed in nonphagocytic cells, ROS play essential roles in processes that are different from pathogen killing, as signal transduction, differentiation, and gene expression. The different outcomes of these events are likely to depend on the specific subcellular site of ROS formation, as well as the duration and extent of ROS production. While excessive accumulation of ROS has long been appreciated for its detrimental effects, there is now a deeper understanding of their roles as signaling molecules. This could explain the failure of the "all or none" pharmacologic approach with global antioxidants to treat several diseases. NADPH oxidase is the first source of ROS that has been identified in macrophages. However, growing evidence highlights mitochondria as a crucial site of ROS formation in these cells, mainly due to electron leakage of the respiratory chain or to enzymes, such as monoamine oxidases. Their role in redox signaling, together with their exact site of formation is only partially elucidated. Hence, it is essential to identify the specific intracellular sources of ROS and how they influence cellular processes in both physiological and pathological conditions to develop therapies targeting oxidative signaling networks. In this review, we will focus on the different sites of ROS formation in macrophages and how they impact on metabolic processes and inflammatory signaling, highlighting the role of mitochondrial as compared to non-mitochondrial ROS sources.

**Keywords: macrophages, reactive oxygen species (ROS), mitochondria, innate immunity, redox signaling, inflammasome, monoamine oxidase, protein oxidation**

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## 1 INTRODUCTION

The immune system orchestrates a complex defensive strategy against pathogens or tissue injury. In vertebrates, two types of immunity are used to protect the host from infections: innate and adaptive. The innate system, which constitutes the first line of defense, is genetically programmed to recognize structures that are broadly shared by invading microbes (named PAMPs, pathogen-associated molecular patterns) and by cell damage (named DAMPs, damage-associated molecular patterns). Cells of the innate immune system include macrophages, dendritic cells, neutrophils, eosinophils, basophils, mast cells and Natural Killer cells. In contrast, the adaptive system, also

referred as the acquired immune system, employs antigen-specific receptors that are specifically developed ("acquired") by lymphocytes during the lifetime of the organism.

Macrophages are large, specialized cells that rapidly recognize, engulf, and destroy pathogens or apoptotic cells. Indeed, the term macrophage is formed by the combination of the Greek terms "makro" meaning big and "phagein" meaning eat. One of the fundamental features of macrophages is their high plasticity, which allows them to respond to stimuli from the complex tissue microenvironment, by changing rapidly their functional profile through a process named "polarization". In fact, they initially adopt a proinflammatory phenotype and then later they acquire an anti-inflammatory profile to repair the tissue damage (1, 2). Due to the complex stimulating network, the process of macrophage polarization in an *in vivo* setting cannot be recapitulated by the static vision of M1-M2 polarization adopted in *in vitro* experiments, reached by stimulation with lipopolysaccharide (LPS)/interferon-γ (IFN-γ) or Interleukin (IL)-4/IL-13 or IL-10, respectively (2, 3). However, macrophages with predominantly proinflammatory properties are commonly referred as M1, whereas those with a pro-fibrotic and anti-inflammatory signature as M2. They are found ubiquitously in tissues as resident cells patrolling their surroundings, thus maintaining tissue integrity. Resident macrophages have different names according to where they function in the body. For instance, macrophages in brain are termed microglia, while in liver they are called Kupffer cells (4). Moreover, in case of tissue damage or infection, monocytes leave the bloodstream to enter the affected tissues and undergo a series of changes to become macrophages.

Reactive oxygen species (ROS) have been known for many years as fundamental for macrophages to kill invasive microorganisms through the oxidative burst mediated by NADPH oxidase (5). However, more recent studies have shown that mitochondrial ROS play essential roles in several innate immune functions, through subtle changes in the intracellular redox state (6).

Oxygen is a highly electronegative element that readily accepts electrons generated by normal oxidative metabolism within cells, thereby producing ROS. The term "ROS" includes superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide ($H_2O_2$), hydroxyl radical and singlet oxygen, which are produced as described in (6). Besides ROS, other endogenous small, reactive signaling molecules include reactive nitrogen species (RNS), such as nitric oxide, as well as hydrogen sulfide and carbon monoxide. Several reviews discuss their roles in macrophage function (7–9), so they will not be considered in the present review.

While ROS have been considered for a long time as dangerous by-products of mitochondrial metabolism, it is now widely accepted that they play crucial roles as signaling molecules, regulating cell growth, differentiation, and apoptosis (10). As discussed extensively in other reviews (5, 6), ROS appear to play different -sometime opposing- roles depending on their subcellular origin and levels. Among ROS, $H_2O_2$ is by far the most prevalent and best studied cellular oxidant and plays a major role in redox regulation of biological activities (11). Similar to other signaling molecules, the intracellular concentrations of $H_2O_2$ are maintained very low (in the range of 1-100 nM) and are tightly regulated (6). They are produced in such a low level to be confined to a restricted subcellular location and to induce signaling pathways, supporting normal physiological processes. This range of concentration must be considered just as an order of magnitude, because it depends on many factors, as cell type, local concentrations, etc. Different stimuli, such as growth factors or chemokines, trigger a physiological increase of $H_2O_2$, which targets specific proteins leading to their reversible oxidation, thereby altering their activity, localization and interactions. These protein modifications contribute to orchestration of various processes in cells and organs, including cell proliferation, differentiation, migration and angiogenesis. On the other hand, high levels of ROS (roughly reaching micromolar concentrations) are likely to become involved in non-specific oxidation of targets, causing damage to macromolecules, impairing their function and triggering stress response mechanisms, as inflammation, fibrogenesis, tumor growth, metastasis and, at higher levels, cell death (6, 12). In this review, we will focus on the different sites of ROS formation in macrophages, the major molecular redox targets, and their related cellular response.

# 2 SOURCES OF ROS IN MACROPHAGES

## 2.1 Cytosolic Sources

### 2.1.1 NADPH Oxidases

NADPH oxidases (NOXs) are a family of transmembrane enzymes specifically dedicated to produce cytosolic ROS (cytROS) (13, 14), mainly located in the plasma membrane (**Figure 1**). NOXs catalyze superoxide formation by transferring one electron from NADPH to oxygen (15). Superoxide can be further converted to $H_2O_2$ either by spontaneous dismutation or catalyzed by superoxide dismutase. So far, seven members of the family have been described (NOX1-5 and Duox1-2) (16), and three of them (NOX1, NOX2 and NOX4) have been identified in phagocytes (17–27). NOX2 is the most well-characterized isoform for its role in phagocytic function. During phagocytosis, the plasma membrane is internalized and becomes the interior wall of the phagocytic vesicle. Next, the $O_2^{\cdot-}$ produced by NOX2 (named "oxidative burst") is released into the vesicle to kill the internalized target. The relevance of cytROS in host immunity has been demonstrated in chronic granulomatous disease (CGD), a genetic disorder characterized by mutations in genes encoding components of the NOX2 complex (28, 29). Patients with CGD are hypersensitive to both bacterial and fungal infections, as their phagocytic cells fail in killing pathogens due to the extremely low oxidative burst during phagocytosis (30, 31). More recently, it has been shown that also NOX4-mediated ROS production is selectively required for the host defense against *Toxoplasma gondii* infection (26).

Besides killing invasive microorganisms, NOX-dependent ROS production influences many metabolic processes and

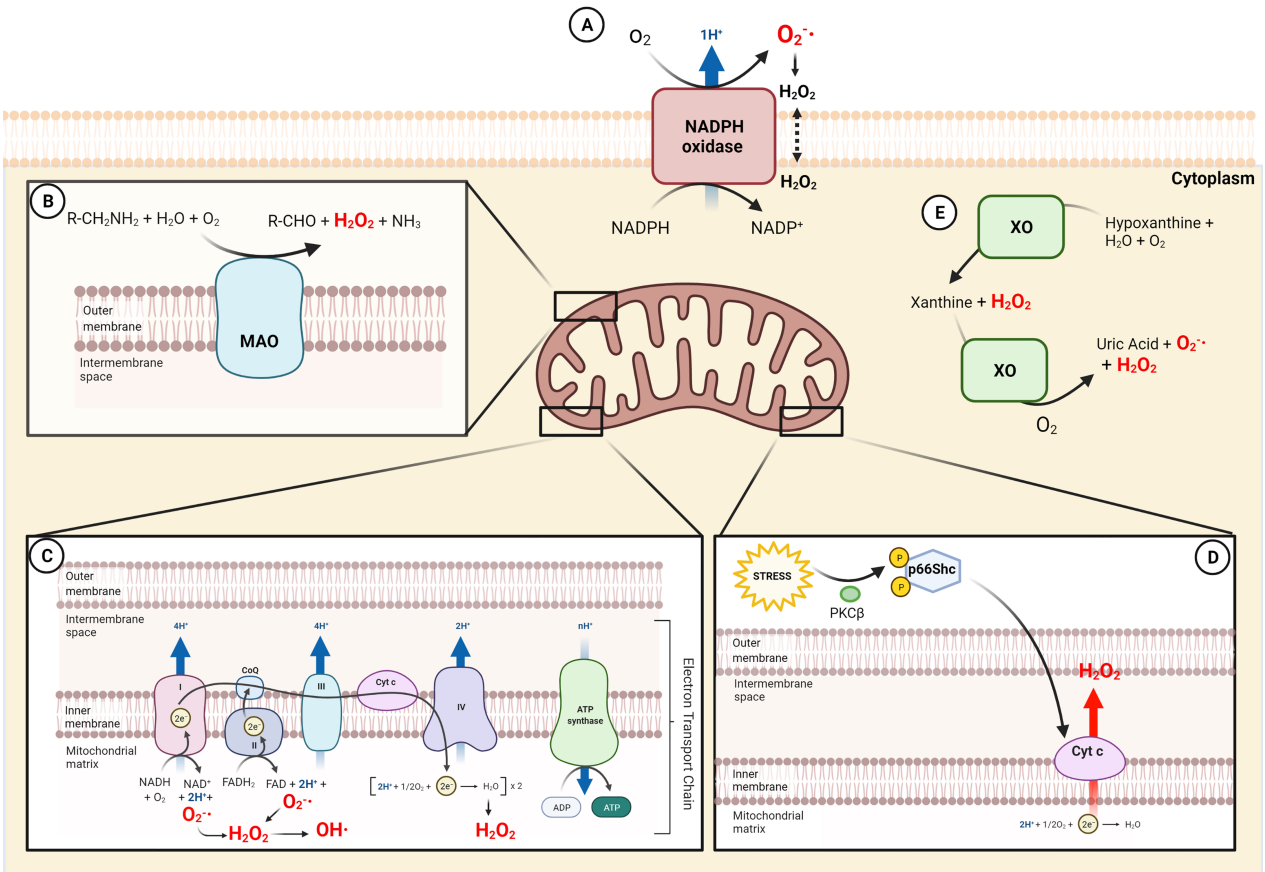


**FIGURE 1** | Sources of ROS in macrophages. **(A)** Generation of $O_2^{-\cdot}$ and consecutive formation of $H_2O_2$ by NADPH oxidase (NOX) in the external cell membrane. **(B)** Production of $H_2O_2$ in the outer mitochondria membrane by oxidative deamination of biogenic and xenobiotic amines by MAO. **(C)** Electron Transport chain in the inner mitochondrial membrane generates $O_2^{-\cdot}$, $H_2O_2$ and OH· in the mitochondrial matrix. **(D)** Cytochrome *c* in the inner mitochondrial membrane produces $H_2O_2$ following p66Shc activation by stress. **(E)** The xanthine metabolism produces $H_2O_2$ and $O_2^{-\cdot}$ by XO in the cytoplasm. $NADP^+$, Nicotinamide adenine dinucleotide phosphate; MAO, monoamine oxidases; CoQ, coenzyme Q; FAD, flavin adenine dinucleotide; Cyt *c*, cytochrome *c*; ADP, adenosine diphosphate; ATP, adenosine triphosphate; PKCβ, protein kinase C β; XO, xanthine oxidase.

disease states. For instance, NOX1 and NOX2 are critical for the differentiation of monocytes to macrophages and the M2-type polarization, as assessed in macrophages from NOX1/NOX2 double knockout mice (19). Interestingly, ROS generated by NOX2 were found to contribute to fatty liver disease (27). Indeed, *Nox2*-deficient mice were protected against hepatic steatosis induced by high-fat diet and insulin resistance (27). Mechanistically, palmitate triggers endocytosis of the Toll-like receptor 4 (TLR4)-MD2 complex, leading to NOX2 activation, ROS generation and proinflammatory cytokine production in hepatic infiltrating macrophages (27). By the way, although palmitate has been considered a TLR4 ligand, a subsequent study demonstrated that it does not directly bind this receptor (32). More recently, NOX1-dependent ROS production has been found to be neurotoxic for microglia located in the retina (33). Its overactivation is mediated by the translocator protein TSPO. Using different NOX-deficient mice, the study shows that the TSPO-NOX1 axis controls the phagocyte-triggered angiogenesis in the eye of a mouse model of age-related macular degeneration, a major cause of blindness in the elderly (33). NOX4 has also been shown to be an inducible source of ROS, driving cell death when monocytes and macrophages were exposed to oxidized low density lipoproteins (oxLDL) (17).

Finally, different studies investigated the impact of NOX in the activation of the NLRP3 inflammasome in macrophages. The NLRP3 inflammasome is a molecular platform activated upon signs of cellular danger (PAMP or DAMP) to trigger innate immune defenses through the maturation of pro-inflammatory cytokines (34, 35). For its activation it requires the adapter protein apoptosis associated speck-like protein containing a CARD (ASC) to activate caspase-1, which cleaves pro-interleukin (IL)-1β and pro-IL-18 in IL-1β and IL-18 (34). The results linking NOX to the NLRP3 inflammasome are controversial, and this could be ascribed to species differences, differential regulation of monocytes and macrophages or redundant NOX enzymes, as discussed (23, 36, 37). Also the localization of NOX4 is quite arguable/disputed, as some studies

found this isoform even in mitochondria, others in the plasma membrane and in the endoplasmic reticulum (6).

### 2.1.2 Xantine Oxidase

Xanthine dehydrogenase is an enzyme that can be converted to its oxygenase form xantine oxidase (XO) upon oxidative stress. Both forms generate uric acid from hypoxanthine or xanthine, but XO also produces ROS (38) (**Figure 1**). The conversion of xanthine dehydrogenase to XO can be due to irreversible proteolytic cleavage, or to reversible sulfhydryl modification (39, 40). Its role has been widely studied for many years, but scarce information is available with respect to the innate immune system. Interestingly, convincing evidence reports that XO is a source of ROS that mediates NLRP3 inflammasome activation in macrophages (41) and that represents a key factor to trigger inflammation against parasitic infection (42).

## 2.2 Mitochondrial Sources

Mitochondria are important sources of ROS, as they are the main oxygen consumers in the cell (43). These organelles produce ROS through various mechanisms, including electron leak from the electron transport chain to oxygen, or as by-products of the catalytic activity of several oxidases (44–46). Interestingly, it has been suggested that also mitochondrial ROS (mtROS) represent an important component of the antibacterial responses, thereby revealing a novel pathway linking innate immune signaling to mitochondria (47). Specifically, the engagement of a subset of Toll-like receptors (TLR1, TLR2 and TLR4) was found to cause the recruitment of mitochondria to macrophage phagosomes and augments mtROS, although the mechanism is still partially unclear (47). Another study highlighted the relevance of mtROS as compared to cytROS. Bulua and Coworkers showed that mtROS are responsible for excessive LPS-driven production of proinflammatory cytokines in cells from patients with an autoinflammatory disorder caused by missense mutations in the type-1 TNF receptor (TNFR1), named TNF receptor-associated periodic syndrome (TRAPS) (48). On the other hand, NOXs are not the source of proinflammatory ROS, as NOX subunits were found to be dispensable for inflammatory cytokine production (48). The authors hypothesized several mechanisms by which TNFR1 mutations enhance mitochondrial respiration, although further research should more specifically address this issue. The endoplasmic reticulum, where mutant TNFR1 resides, can provide signals to activate mitochondrial respiration (49). Moreover, mutant TNFR1 may increase activation of the riboflavin kinase, which can associate with TNFR1 (50), possibly leading to enhanced charging of FAD-dependent enzymes in the mitochondria.

### 2.2.1 Electron Transport Chain

The electron transport chain (ETC) is a series of electron-carrier proteins located in the mitochondrial inner membrane (**Figure 1**). It transfers electrons from the reduced coenzymes NADH and $FADH_2$, generated by catabolic processes, to molecular oxygen. Thanks to this process, three of the four protein complexes pump protons across the mitochondrial inner membrane to maintain the protonmotive force driving ATP synthesis. A physiological consequence of the electron transfer is the generation of mitochondrial ROS (mtROS). In fact, the electron leak from complexes I, II, and III mediates the one-electron reduction of oxygen to superoxide ($O_2^{\bullet -}$), which can then be rapidly converted to $H_2O_2$ by manganese superoxide dismutase (MnSOD) within the mitochondrial matrix. $H_2O_2$ can then freely diffuse in the cell and trigger thiol oxidation of proteins.

mtROS can be also produced at complex I through *reverse electron transfer (RET)*. This process, observed *in vitro* in the sixties of the last century (51, 52), has been considered of uncertain physiological relevance for many years. More recently, several studies highlighted that RET at complex I is a process underlying mitochondrial redox signaling in physiological and pathological conditions (45, 53–56). It occurs when electrons flow back through complex I, in contrast to conventional forward transport, because of elevated mitochondrial membrane potential coupled to highly reduced coenzyme Q. Thus, the electrons can reduce $NAD^+$ to NADH and drive superoxide formation. Indeed, in macrophages stimulated with LPS, Mills et al. showed that the accumulation of succinate, that is oxidized by complex II (succinate dehydrogenase), results in mtROS production, seemingly from RET at complex I (45).

### 2.2.2 Monoamine Oxidases

A relevant mitochondrial-specific source of ROS is monoamine oxidase (MAO), although its impact on inflammation has been quite overlooked. MAO is located in the outer mitochondrial membrane and catalyzes the oxidative deamination of neurotransmitters (i.e. catecholamines) and dietary amines, generating aldehydes, ammonia and $H_2O_2$ (57) (**Figure 1**). The two isoforms MAO-A and MAO-B differ for substrate specificity and inhibitor sensitivity. MAO-A has greater affinity for hydroxylated amines, i.e. serotonin and noradrenaline, whereas MAO-B has greater affinity for non-hydroxylated ones, i.e. β-phenylethylamine. Notwithstanding, they show similar affinity for dopamine and tyramine. MAO physiologic role is well established in the central nervous system: it terminates neurotransmitter signaling and, by doing this, it generates $H_2O_2$ that is constantly removed by endogenous scavengers (57). On the contrary, in pathological conditions, the increased activity of the enzyme overcomes the cellular antioxidant defenses, altering the redox homeostasis and eliciting deleterious effects, as in muscular dystrophy and cardiac injury (58–62). Only few studies have characterized the role of MAO in innate immunity. It has been shown that MAO-A is upregulated by LPS or by IL-4/IL-13 in phagocytic cells (63–66). These studies also suggest that IL-13 and IL-4 induced MAO-A-mediated ROS generation through Jak signaling pathways (65).

Few years ago, a study from Tschopp's group highlighted the crucial role of mtROS in NLRP3 inflammasome activation, although the source of ROS production was unclear (65, 67). Recently, it has been shown that $H_2O_2$ produced by MAO-B plays a non-redundant role in sustaining NLRP3 inflammasome activation (68) in human and murine macrophages. Mechanistically, MAO-B-dependent ROS formation causes

mitochondrial dysfunction and NF-κB activation, resulting in NLRP3 and pro-IL-1β overexpression. Both *in vitro* and *in vivo*, MAO-B inhibition by the clinical grade drug rasagiline prevents IL-1β secretion, and MAO-B deficient mice display an impaired response to LPS-mediated endotoxemia (68).

Remarkably, two interesting studies highlight the critical role of MAO in macrophages, considering the enzyme as a catecholamine consumer and not as a ROS producer. Briefly, macrophages from adipose tissue of aged mice displayed upregulation of MAO-A in a NLRP3 inflammasome-dependent manner. The enhanced activity of MAO-A increases catecholamine catabolism, thereby dampening the activation of lipolytic signaling in adipocytes as this process depends on noradrenaline levels (69). In the same context, MAO-A was identified in a subset of cells called macrophages associated with sympathetic neurons (SAMs) that work in the clearance of noradrenaline, acting as a sink (70). Moreover, SAMs were increased in two models of obesity and contributed to the disease by excessive import and metabolism of noradrenaline (70, 71).

#### 2.2.3 P66shc

p66Shc is a cytosolic adaptor protein that regulates the cellular redox state and apoptosis (72, 73). Oxidative stress activates protein kinase C-β (PKCβ), which phosphorylates p66Shc. As a result, p66Shc translocates to mitochondria, where it generates $H_2O_2$. Indeed, this redox enzyme utilizes reducing equivalents of the mitochondrial ETC through the oxidation of cytochrome *c* to catalyze the partial reduction of molecular oxygen (72). Mice lacking p66Shc are long-lived, and their cells are both resistant to oxidative stress and produce less ROS. A few studies characterized p66Shc in the immune system and were mainly focused on its role in the macrophages of the atherosclerotic lesions induced by high-fat diet or diabetes (74, 75). Chronic high-fat diet was reported to increase the atherosclerotic lesion area more in wild-type than *p66Shc* knockout mice. Early lesions from *p66Shc* knockout mice had fewer macrophage-derived foam cells as compared to those from wild-type mice (74). A cross-talk between p66Shc and NOX has been observed in murine macrophages, as NOX activation is defective in p66Shc-deficient mice, leading to decreased superoxide production (76).

## 3 TARGETS OF ROS IN MACROPHAGES

It is widely accepted that physiological levels of ROS can cause reversible post-translational modifications in proteins to regulate signaling pathways. More in detail, $H_2O_2$ can oxidize thiol groups (-SH) on cysteine residues to form sulfenic acid (-SOH), which can react with GSH to become glutathionylated (-SSG), or with adjacent thiols to form a disulfide bond (-SS-) (6, 77). Cysteine thiols can be considered as redox sensors, as they are particularly sensitive to oxidants. The reactivity of thiols for ROS is limited to the cysteinyl residues placed in sites that enable the formation of the thiolate form ($-S^-$), which is more nucleophilic and susceptible to oxidation (6). These modifications can change the activity of the target proteins, thus altering their function and their downstream signaling pathway and metabolism (78). Supraphysiological concentration of ROS leads to oxidative stress, a state of imbalance between ROS production and ROS removal, which can be either due to increased ROS formation or to reduced antioxidant defenses.

In this review, we summarize several examples of signaling targets for ROS that have been identified in macrophages, as the list of targets is too extensive to be covered exhaustively (6). Importantly, cysteine oxidation networks have been recently become accessible (79) and provide a quantitative tissue-specific overview of the redox-regulated proteome, called the Oximouse dataset (79).

### 3.1 Nrf2/Keap1 Complex

Nrf2 (nuclear factor-erythroid 2 p45-related factor 2) is the master regulator of the antioxidant response: it is responsible for maintaining the redox homeostasis under oxidative stress by regulating the expression of detoxifying enzymes involved in glutathione, NADPH and thioredoxin systems (including GCLC, GCLM, NQO1, G6DH, TRX and HO-1) (12, 80, 81). Under basal conditions, Nrf2 is constitutively degraded by the proteasome, as its cytosolic repressor Keap1 (Kelch-like ECH-associated protein 1) enables the Cul3-Ring-box 1 E3 ubiquitin ligase to ubiquitinate it. Oxidative stress induces the oxidation of Cys151, Cys273 and Cys288 in two domains of the negative regulator Keap1, inducing its conformational change and disruption of the Nrf2/Keap1 interaction (82). This results in the stabilization of Nrf2, which translocates to the nucleus and binds specific DNA sequences, the Antioxidants Response Elements (ARE) (83–85). The role of Nrf2 in macrophages is still controversial, as both anti-inflammatory and pro-inflammatory mechanisms have been described, as summarized here below. Several studies demonstrate that the Keap1/Nrf2/ARE signaling pathway attenuates inflammation. Nfr2 deletion causes exacerbation of inflammation in different experimental murine models, such as septic shock and emphysema (86–88), and antioxidant treatments reduce these effects. A recent study reports that murine macrophages achieve self-protection against oxidative stress through the Mst-Nrf2 axis (89). The kinases Mst1/2 sense ROS and maintain the cellular redox balance by modulating Nrf2 stability. Mechanistically, both phagosomal and mtROS activate Mst1/2, which phosphorylate Keap1, thereby blocking Nrf2 ubiquitination and degradation to protect cells against oxidative damage and to maintain the phagocytosis properties (89). Nrf2 was found to inhibit the NLRP3 inflammasome assembly by buffering ROS and controlling the expression of thioredoxin, which inhibits a protein necessary for NLRP3 complex stabilization, the thioredoxin-interacting protein (TXNIP) (90) (see 3.6 *Thioredoxins and ASK1* for further details). On the other hand, other studies provide evidence for a proinflammatory role. Indeed, Nrf2 plays an essential role in ROS-mediated inflammasome activation, as Nrf2-deficient macrophages show a reduced formation of ASC specks and IL-1β release in response to NLRP3 and AIM2 inflammasome stimuli (91). ASC is an

adaptor protein which assembles into a large protein complex, termed "speck", upon inflammasome activation. Hence, ASC speck formation is commonly used as a simple upstream readout for inflammasome activation (92).

The paradoxical role of Nrf2 might be related to the kind of inflammatory stimuli and/or the timing of activation. In fact, the early activation of Nrf2 triggered by antioxidant treatments can enhance the antioxidant response, reduce ROS levels and induce an anti-inflammatory signature, whereas the late activation can mainly contribute to the NLRP3 assembling, such as the one triggered by cholesterol crystals.

Despite its strong association with redox biology, the activation of Nrf2 by mtROS induces also the overexpression of macrophage-specific genes that are not classified as anti-oxidative stress-response genes, such as the gene encoding MARCO, a scavenger receptor required for bacteria phagocytosis (93). Furthermore, Nrf2 can inhibit the expression of proinflammatory cytokine genes in a redox-independent manner. Indeed, Nrf2 was found to inhibit the recruitment of RNA polymerase II onto the proinflammatory cytokine gene loci for Il-1β and Il-6 (94).

## 3.2 HIF Pathway

Hypoxia Inducible factors (HIFs) are transcription factors, consisting of an oxygen-labile subunit (HIF-α) and a constitutively stable subunit (HIF-β), which play pivotal roles in inducing cellular responses to hypoxia and in regulating immune cell effector functions (95, 96). They form a heterodimeric complex, which binds to hypoxia-responsive elements (HREs), thus activating the transcription of their target genes and promoting a metabolic and functional cell reprogramming (97). In homeostatic conditions, HIF is hydroxylated by prolyl hydroxylase (PHD), an enzyme belonging to the 2-oxoglutarate (2-OG)-dependent dioxygenase (2-OGDD) family, which catalyzes the conversion of 2-OG to succinate and employs molecular oxygen for HIF hydroxylation. The hydroxylation of HIF drives its ubiquitylation by the E3 ligase von Hippel–Lindau tumour-suppressor protein (VHL) and its consequent degradation by the proteasome (5). Upon LPS stimulation, macrophages reprogram their metabolism towards glycolysis, thereby driving ROS generation which can sustain hypoxic adaptation by HIF stabilization (3, 45, 98) and concomitantly support the expression of cytokines, such as IL-1β *via* HIF-1α. The crucial role of ROS is documented by a strong body of evidence. Limiting ROS production by uncoupling mitochondria or by expressing the alternative oxidase (AOX) inhibits the inflammatory phenotype of macrophages (45). Moreover, ROS contribute to HIF-1α stabilization by (I) diverting the PHD substrate 2-OG toward a non-enzymatic decarboxylation (99), and (II) oxidizing the PHD cofactor $Fe^{2+}$ to $Fe^{3+}$ (100). Finally, ROS act as inhibitor of Factor Inhibiting HIF (FIH), an asparaginyl hydroxylase belonging to the 2-OGDD family. FIH hydroxylation, occurring in a different site from PHD hydroxylation (101), impairs HIF-1α function by reducing its C-terminal transactivation domain activity (101). It is worth noting that HIF asparaginyl hydroxylation (OH) differs from the prolyl OH in the fact that is more sensitive to low concentrations of $H_2O_2$ than prolyl OH, whereas in moderate hypoxia asparaginyl OH is less effectively inhibited than prolyl OH. This suggests that hypoxia and ROS can provide different levels of regulation of HIF transcriptional output (101). In support of this finding, although in cancer cell lines, mtDNA depletion is sufficient to prevent HIF-1α stabilization under hypoxia (102). In bone-derived murine macrophages mtROS generated by spermidine activate AMP-activated protein kinase (AMPK), which in turn enhances mitochondrial function, and upregulates HIF-1α (103). Moreover, HIF-1α reduces mitochondrial mass through mitophagy, thus limiting oxygen consumption. MtROS target SDH subunit A (SDHA), leading to the inhibition of its enzymatic activity, which in turn stabilizes HIF-1α and causes the subsequent, sustained expression of IL-1β together with TCA intermediates accumulation (104).

HIF-1α mediates the expression of genes encoding for glycolytic enzymes, for the glucose transporter GLUT1 (98), and the pyruvate dehydrogenase kinase isoform 1 (PDK1) in macrophages (105, 106). When HIF1 is stabilized, PDK activity inhibits the pyruvate dehydrogenase complex, limiting the flux of pyruvate into the tricarboxylic acid (TCA) cycle. This attenuates mitochondrial respiration through ETC flux, preventing mtROS production from overpowering the antioxidant endogenous defense in hypoxic conditions (107, 108). In this scenario, PDK1 activation might also represent a strategy to divert glucose from glycolysis to the pentose phosphate pathway, leading to a higher $NADPH/NADP^+$ ratio that might prevent or compensate for uncontrolled oxidative stress, which could be harmful. Indeed, LPS lethality in mice can be prevented by limiting ROS production (45). PDK activity can also be inhibited by mtROS, providing a regulatory flexibility that is functionally important in the migration setting of macrophages (109).

Besides PHD, ROS can also inhibit the activity of Jumonji domain-containing histone demethylase (JMJD), a family of $Fe^{2+}$-dependent 2-OG oxygenases essential for epigenetic reprogramming in macrophages through histone demethylation. Remarkably, the increase in the succinate/2-OG ratio inhibits PHD and JMJD function activating HIF-1α and sustaining the glycolytic switch and proinflammatory phenotype (110). In this way the HIF-ROS axis represents a central functional cycle of mutual support where ROS production is a mediator of hypoxia adaptation, by translating oxygen limitation into transcriptional regulation for a metabolic reprogramming.

More recently, it has been described that SARS-CoV2 infection triggers mtROS production in monocytes, and this induces HIF-1α stabilization and consequently promotes glycolytic reprogramming, thereby enhancing the viral replication (111). SARS-CoV2 infection induced downregulation of several proteins of the ETC, such as NDUFV1 (complex I), SDHA (complex II), UQCRC2 (complex III), limiting oxygen consumption rate (111). Defects of the mitochondrial respiratory chain have been related to increased mtROS levels (45), although the precise mechanisms linking infection and mtROS production are still to be defined.

## 3.3 NF-κB Pathway

NF-κB is a transcription factor that plays a crucial role in inflammatory and immune responses (112). It displays a

plethora of modulatory mechanisms due to the different DNA binding affinities of their homo- and hetero-dimeric complexes emanating from the five monomers (RelA/p65, RelB, cRel, NF-κB1 p50, and NF-κB2 p52). This heterogeneity is further increased by interactions of the NF-κB dimers with other transcription factors (112). The heterodimeric complex p50/p65 is one of the better characterized during the inflammatory response (113). NF-κB in the cytosol is inactivated by binding to the regulatory protein IkBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha). Inflammatory stimuli drive the phosphorylation of the IKK complex, consisting of two catalytic (IKKα and IKKβ) and one regulatory subunit (NEMO or IKKϒ), the latter acting as scaffold protein for IKKβ activity. The activated complex phosphorylates IκBα, marking the protein for proteasome-linked degradation (114). NF-κB is then free to translocate to the nucleus and start the transcription of several genes, including inflammatory and antiapoptotic genes. The mechanisms linking ROS formation to NF-κB activation are multiple. In macrophages, mtROS are known to mediate IKK complex activation by forming a disulfide bridge between Cys54 and Cys347 on NEMO, which is crucial for IKK complex activation (115). Under proinflammatory stimulus, the IKK complex can be activated by ROS produced by the GTP-binding protein Rac1 (116), leading to signal transduction pathways that contribute to TNF-α secretion. In line with this finding, ROS effects can be suppressed by SOD, reducing the pro-inflammatory immune responses by blocking the p38-MAPK/NF-κB signaling activation (117).

NF-κB is also involved for different transduction outcomes related with anti-inflammatory response. This is particularly evident in the context of the tumor microenvironment, in which ROS formation activates NF-κB signaling, which binds the *Pdl1* promoter in a transcriptional specific manner, leading to PD-L1 expression and release of immunosuppressive chemokines. Indeed, the described ROS-mediated NF-κB activation does not induce expression of the classical NF-κB target IL-6 (118). Similarly, in the context of colitis, mtROS lead to induction of NF-κB signaling responsible of a protective effect associated to the recruitment and polarization of intestinal macrophages to the M2 anti-inflammatory phenotype (119).

Notably, NF-κB is involved also in hypoxic conditions, and evidence of a crosstalk between NF-κB and HIF-1α is growing. PHD inhibition regulates the activity of IKKβ, inducing the nuclear translocation of NF-κB (120). Conversely, hypoxia-mediated NF-κB induction controls HIF-1α activity in macrophages, enhancing the production of pro-inflammatory cytokines and chemokines to sustain the host defense response (121). The link between NF-κB and HIF-1α is further underlined by the finding that HIF-1α promoter contains a NF-κB binding site that can trigger HIF-1α upregulation under conditions of NADPH oxidase-mediated ROS formation (122).

The crosstalk between HIF and NF-κB pathways is extensive, intensive (123) and bi-directional (124). In fact, they are reciprocally regulated (124). An additional level of functional crosstalk between HIF and NF-κB includes common activating stimuli, shared regulators and target genes (123). The overlap of common regulators between HIF and NF-κB consequently finds functional involvement of HIF in processes in which NF-κB is involved, such as infection and inflammation. Many important genes are regulated by HIF and NF-κB (123), including cytokines and chemokines, such as TNF-α, IL- 1β and IL-8. In addition, cell death related proteins, such as Noxa and BNIP3, and other important cellular proteins such as PKM2, Tert, Cyclin D1, and Cox-2 are also shared HIF and NF-κB targets. However, it is not known whether these genes are targeted by these transcription factors at the same time or independently of each other (123).

Moreover, HIF and NF-κB synergistically respond against pathogens. In fact, it has been demonstrated that macrophages, infected by Gram-negative and Gram-positive bacteria, are characterized by a defective HIF-1α expression following the ablation of IKKβ, essential regulator of NF-κB activity (121, 125).

## 3.4 Jak/STAT Pathway

Signal transducer and activator of transcription (STAT) proteins are a family of transcription factors that are essential for the cellular response to cytokines and growth factors. STATs are latent in the cytoplasm under resting conditions. When extracellular stimuli, such as cytokines, bind to specific cell-surface receptors, they activate the tyrosine kinases Jak ('Janus kinase'), that phosphorylates STAT proteins, thereby allowing translocation to the nucleus to drive transcription of several chemokines and cytokines. The JAK-STAT signaling pathway is fundamental for the immune system (126–129). So far, seven members of the family (STAT1-4, STAT5A, STAT5B, and STAT6) have been identified. STATs are fundamental in the inflammatory/anti-inflammatory response, like the antiviral response through interferon production, and in wound healing (130, 131). The JAK-STAT pathway plays an essential role in macrophage polarization: STAT1 shifts macrophages towards a pro-inflammatory M1 profile activated by interferon gamma (IFN-γ), whereas STAT6 is associated to an M2 anti-inflammatory profile through IL-4. The role of ROS during STAT activation in macrophages is still under scrutiny. A positive feedback in the ROS-p38MAPK-STAT1 axis has been described, as $STAT1^{-/-}$ mice showed impairment in p38MAPK activation in a ROS-dependent manner (132). As a further suggestion of a cooperative role between ROS and STAT1 activation, in NOX-deficient diabetic mice the reduced ROS levels were found to impair STAT1 activation, and to increase STAT6 activation, thereby promoting a M2 signature during diabetes progression (133). On the other hand, the $H_2O_2$ produced by Cu-Zn SOD activity in alveolar murine macrophages was found to activate STAT6 by redox regulation of a critical cysteine during the polarization toward M2-like macrophages (134). Taken together, these findings suggest that further studies will be warranted to understand the tight crosstalk between ROS and STAT, considering the sources and the kind of ROS which are involved.

## 3.5 STING Pathway

The Stimulator of Interferon Genes (STING) pathway senses cytosolic double-strand (ds) DNA, that is a sign of microbial

infection, cell injury or nuclear DNA damage (135). Cytosolic dsDNA triggers the activation of the cyclic-GMP-AMP-synthase (cGAS), leading to endogenous generation of cyclic GMP-AMP (cGAMP), a unique second messenger. cGAMP binds to and activates the endoplasmic reticulum transmembrane receptor STING, finally resulting in the production and release of type I interferons (IFN), which are potent anti-viral and anti-cancer cytokines (136). On the other hand, the hyperactivity of STING pathway has been implicated in several debilitating autoimmune syndromes (137–139) (i.e. systemic lupus erythematosus) and in acute and chronic inflammation (140).

STING forms a domain-swapped homodimer in the absence of ligands, whereas, upon cGAMP binding, it undergoes extensive conformational rearrangements, leading to oligomerization (141). Recently, a structural analysis by Ergun et al. demonstrated that STING polymerization is necessary for its activation through the formation of intermolecular disulfide bonds *via* Cys148 (142). Moreover, the increase of mtROS led to cGAS-STING induction of type I IFN (143, 144). Controversially, during herpesvirus infection ROS were found to dampen the type I IFN production in a STING-dependent manner (145). To try to reconcile these findings, the authors speculated that the differences could be ascribed to the amount of ROS, as cysteines can be susceptible to different post-translation modifications, which can either activate or inhibit protein function. More in detail, high ROS levels could oxidize STING thus preventing its polymerization and interferon production, whereas lower levels can promote its assembly. Thus, further studies will be warranted to elucidate how the post-translational modifications of STING by redox regulation affect the innate immune responses against DNA viruses, especially to identify novel immunotherapy targeting IFN production.

### 3.6 Thioredoxins and ASK1

Thioredoxins (TRXs) are small proteins that represent a key protection system against oxidative stress through their disulfide reductase activity. TRXs contain two redox-active cysteines in a Cys-X-X-Cys motif, that can be reversibly oxidized to keep intracellular redox balance (146). Besides the reducing activity, TRXs are also important components of redox signaling pathways. Indeed, TRXs control the activity of several proteins by direct physical interaction (146). For instance, TRX binds the apoptosis signal-regulating kinase 1 (ASK1), thus forming an inactive TRX-ASK1 complex. An increase of intracellular ROS induces disulfide bond formation in TRX. Such a conformational change allows ASK1 release, which activates p38MAPK and NF-κB pathways, and triggers a cell death program (147–149). This axis is supported in macrophages, as LPS stimulation, in a Myd88- dependent manner, induces the production of $H_2O_2$ to activate NF-κB (148). Remarkably, during tuberculosis infection (150) the recognition of tuberculin protein by TLR2 triggered an increase in ROS production that enhanced a positive feedback burst of ROS by TRX-ASK1-p38 activation (150). Another mechanism, described in microglia, indicates that ASK1 activation occurs by sensing extracellular ATP through the P2X7 receptor, which triggers ROS generation leading to TRX-ASK1 release (151). Furthermore, TRX binds to the thioredoxin-interacting protein (TXNIP). TXNIP activation appears to be essential for NLRP3 assembling by different inflammasome agonists, such as monosodium urate crystals (MSU), ATP or high concentration of glucose (152). These stimuli increase ROS production that allows TRX cysteine oxidation, inducing the breakdown of TRX-TXNIP interaction, and allowing TXNIP to bind NLRP3 to stabilize the inflammasome assembly (152). Moreover, endoplasmic reticulum stress induces ROS-mediated IRE1α activation, that increases the TXNIP mRNA stability, enhancing the NLRP3 activation in sterile inflammation (153).

## 4 CONCLUSIONS AND PERSPECTIVES

The studies summarized in this review highlight the crucial and versatile functions of cytosolic and mitochondrial ROS in macrophages. The signaling and damaging properties of ROS can drive the inflammatory response, as well as the diseases resulting from chronic or overwhelming inflammation. The redox balance depends on many parameters, including the levels and the compartmentalization of ROS, their specific sources and subspecies and the specificity/selectivity for their targets.

The great impact of oxidative stress in many diseases explains the enormous number of studies and clinical trials targeting ROS for therapeutic purposes. However, non-selective antioxidants at high doses did not prove effective in either preventing or treating disease processes. This is probably to be ascribed to the disruption of crucial intracellular redox signaling. Even worse, clinical trials have showed harmful effects of antioxidants (154). Indeed, the physiological relevance of $H_2O_2$ signaling was still unclear when most of these trials were performed (6, 155). It is now widely accepted that ROS are part of a signaling network with different sources and targets. Their different subcellular compartmentalization and expression suggest the presence of multiple hot spots of ROS with different roles, rather than a homogenous intracellular redox level. The expanding knowledge about the pleiotropy of ROS signaling requires that therapeutic interventions use strategies aimed at addressing the specific disease-relevant mechanisms without disrupting other crucial signaling pathways.

With this respect the development of novel therapeutic approaches targeting ROS will require to identify and dampen the main sources of deleterious ROS relevant for a specific disease, without altering the vital physiological sources of ROS and their downstream signaling pathways. The clinical status of these mechanism-based redox therapies is summarized in (154). An example is the inhibition of specific NOX isoforms or the use of Nrf2 agonists enhancing the expression of endogenous antioxidant enzymes at their physiological sites. Our studies support the relevance of targeting the mitochondrial enzyme MAO to counteract inflammatory diseases for several reasons. Indeed, MAO inhibitors, for which the mechanism of action, pharmacodynamics and pharmacokinetics are well established (they are approved drugs from Parkinson's disease), block the formation of a specific subset of mitochondrial ROS relevant in pathological conditions, thus preventing mitochondrial

dysfunction. More research targeting specific ROS sources and defined mechanisms are strongly awaited.

## AUTHOR CONTRIBUTIONS

MC, RS-R, and AC: conceptualization, writing original draft, reviewing, and editing. AV: supervision, reviewing. IS and MF: supervision, literature searching, reviewing, and editing. FV: supervision, reviewing, and editing the figures. All authors contributed to the article and approved the submitted version.

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# GM-CSF Nitration Is a New Driver of Myeloid Suppressor Cell Activity in Tumors

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Reactive oxygen species, including RNS, contribute to the control of multiple immune cell functions within the tumor microenvironment (TME). Tumor-infiltrating myeloid cells (TIMs) represent the archetype of tolerogenic cells that actively contribute to dismantle effective immunity against cancer. TIMs inhibit T cell functions and promote tumor progression by several mechanisms including the amplification of the oxidative/nitrosative stress within the TME. In tumors, TIM expansion and differentiation is regulated by the granulocyte-macrophage colony-stimulating factor (GM-CSF), which is produced by cancer and immune cells. Nevertheless, the role of GM-CSF in tumors has not yet been fully elucidated. In this study, we show that GM-CSF activity is significantly affected by RNS-triggered post-translational modifications. The nitration of a single tryptophan residue in the sequence of GM-CSF nourishes the expansion of highly immunosuppressive myeloid subsets in tumor-bearing hosts. Importantly, tumors from colorectal cancer patients express higher levels of nitrated tryptophan compared to non-neoplastic tissues. Collectively, our data identify a novel and selective target that can be exploited to remodel the TME and foster protective immunity against cancer.

**Keywords: reactive nitrogen species, cytokines, immunosuppression, post-translational modification, tumor microenvironment**

**Abbreviations:** BM, bone marrow; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-, interleukin; Mo-MDSC, monocytic MDSCs; TAMs, tumor-associated macrophages; MDSCs, myeloid derived suppressor cells; PMN-MDSCs, polymorphonuclear myeloid derived suppressor cells; PTM, post-translational modifications; RNS, Reactive Nitrogen Species; TANs, tumor associated neutrophils.

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## INTRODUCTION

Host immune system can intercept, recognize and neutralize tumor cell clones. Following this paradigm, cancer immunotherapy has become a tangible opportunity for the treatment of different cancers as the metastatic melanoma, lung cancer and colorectal cancer (1, 2). In 2011, the US Food and Drug Administration (FDA) approved the first checkpoint-inhibitor drug (Ipilimumab) and nowadays a large number of clinical trials are ongoing worldwide. Nevertheless, immunotherapy still has obvious complexity and uncertainty. Indeed, the efficacy of such treatments is still incomplete mainly due to the unforeseen fatal side effects caused by the immune system overactivation and, more to the strong immunosuppression promoted by the engrafting tumors in the hosts. Seminal evidence indicates that tolerogenic and immunosuppressive circuits in tumor-bearing hosts co-opt host immune system finally dampening anti-tumor immunity. In particular, high frequency of myeloid immunosuppressive cells is tightly connected with tumor promotion, metastasis and poor prognosis in several type of cancers. Indeed, one of the key suppressive mechanisms tailoring cancer progression is the appearance of an unbalanced myelopoiesis in tumor-bearing hosts (3). This process leads to the expansion and differentiation of distinctive myeloid cell subsets that are principally involved in promoting T cell unresponsiveness toward tumor antigens (4, 5). The aberrant myelopoiesis occurring during tumor progression produce different types of immunosuppressive cells like tumor-associated macrophages (TAMs), tumor associated neutrophils (TANs) and myeloid derived suppressor cells (MDSCs). MDSCs are deeply investigated due to their strong ability to directly inhibit T cell functions and promote tumor progression. Early studies in tumor bearing mice described MDSCs as a heterogeneous population of myeloid cell lineage that is characterized by the concomitant expression of the surface markers CD11b and Gr-1 (6, 7). On the basis of phenotypical and functional features, MDSCs are currently arranged in two main subsets: polymorphonuclear MDSCs (PMN-MDSCs) and monocytic MDSCs (Mo-MDSC). In mice, PMN-MDSCs are phenotypically defined as $CD11b^{+}$, $Ly6C^{low/-}$, $Ly6G^{+}$ cells while Mo-MDSCs are $CD11b^{+}$, $Ly6G^{-}$, $Ly6C^{hi}$ (8). MDSCs were found in all types of cancer to a different extent. Nonetheless, their frequency and phenotype are extremely variable in human malignancies thus increasing the complexity of their characterization. Key drivers for MDSC generation and expansion are two cytokines: the GM-CSF and IL-6. Indeed, clear evidence in transplantable tumor models unequivocally connects GM-CSF to the systemic expansion of an immature myeloid population (MDSCs) that actively suppresses anti-tumor T cell immunity (9–11). *In vivo* data confirmed that tumor-derived GM-CSF orchestrates the intra-tumoral accumulation of highly immunosuppressive $CD11b^{+}Gr\text{-}1^{+}$ myeloid cells in a spontaneous pancreatic ductal adenocarcinoma (PDAC) model (12). Nonetheless, other experimental evidence paradoxically indicates that GM-CSF acts as a potent immunostimulatory product and its efficacy as vaccine-adjuvant has been demonstrated in different regimens (13, 14). Reasonable explanations for this antithetical conduct could be found in a quantitative difference in location and dosage of the very same cytokine in tumors (15) or most likely, in a qualitative divergent tumor-related mechanism. Thus, GM-CSF can act in cancer as corrupted cytokine, co-opted by the tumor itself to sculpt an immunosuppressive environment ultimately establishing its immune privilege (16).

Notably, the release and overload of inflammatory reactive species are prominent features of cancer. In particular, high levels of nitrated proteins have been detected in several human tumors, including prostate, colon, liver, breast, and ovarian neoplastic tissues (17). Our group previously discovered a novel mechanism of tumor escape based on the post-translational modification (PTM) of intra-tumoral chemokines, which can be pharmacologically targeted to improve the efficacy of cancer immunotherapy. Indeed, the nitration of the inflammatory chemokine CCL2 within the tumor microenvironment (TME) altered its chemoattractant properties finally limiting T cell entry into the primary tumor lesion (18). Although we originally focused on a single protein, other cytokines and chemokines should be target of such modification contributing to shape anti-tumor immunity. In this manuscript, we reasoned that the cytokine GM-CSF, which is abundantly expressed in several human cancers, represents a possible target for ROS/RNS-induced PTM in the TME. We found that RNS significantly altered the GM-CSF activity in tumor-bearing hosts by causing protein nitration at a specific site, a tryptophan, that we identified. Moreover, we revealed the molecular signature activated by the nitrated cytokine in myeloid cells, which is responsible for their enhanced immunosuppressive phenotype and activity. Finally, we provided evidence that GM-CSF nitration *in vivo* significantly affects the immune landscape at the tumor site. Importantly, GM-CSF nitration in the Tryptophan residue can be also found in human colorectal cancer specimens, where we observed higher levels of both GM-CSF and nitrated-Tryptophan in comparison to the surrounding non-transformed ones.

Collectively, our data identify the nitrated-GM-CSF as a novel player of tumor-related immunosuppression, which can be pharmacologically targeted in novel immune-based strategies against cancer.

## METHODS

### Mice

C57BL/6 and OT-I (C57BL/6- Tg (Tcra Tcrb) 1000Mjb/J mice were purchased from Charles River Laboratories. All experiments were performed with mice of 8 weeks maintained in the animal facilities of the Venetian Institute of Molecular Medicine (VIMM). Experiments were performed according to the guidelines approved by the local ethics committee.

### Plasmids

The encoding sequence for GM-CSF were taken from pubmed and obtained by gene synthesis from GenScript. This sequence codifies the full length of GM-CSF plus the 6XHis tag introduced at the C-terminus after AgeI restriction site. To allow further cloning, two cloning sites were introduced at 5' and 3'. The plasmid obtained from GeneScript were cloned HINDIII/NHE

into pcDNA3.1-hygro (Invitrogen). To generate the mutant version of GM-CSF, a site-specific mutagenesis approach was used with the following oligos:

mut W>L for: 5'-ccatcactgtcacccggcctCTgaagcatgtagaggccatc-3'
mut W>L rev:5'-gatggcctctacatgcttcAGaggccgggtgacagtgatgg-3'.

## Cell Line

MCA-203 fibrosarcoma cell line was maintained in DMEM (Lonza) supplemented with 10% heat-inactivated FBS (Gibco), 2 mM L-glutamine (Gibco), 10 mM HEPES (Lonza), 55 uM 2-mercaptoethanol (Gibco), 150 U/ml streptomycin, 200 U/ml penicillin obtained from the American Type Culture Collection (ATCC). Two additional cell lines were generated by the stable transfection with either electroporation or the usage of Lipofectamine 2000 (Invitrogen) of MCA cell line with the plasmid encoding either GM-CSF or its mutant version GM-CSF W30L. Transfected cells were selected and thus culture in the media used for MCA-203 supplemented with 0,3 ug/ml Hygromicin (Invitrogen).

## Tumor Challenge

C57BL/6 mice were injected on the right flank with $10^6$ MCA203-GM cells or MCA203-W30L cells. Tumors were measured every 2 days by a digital caliper. At day 21 post-tumor challenging, mice were euthanized, and their tumors explanted and processed for flow cytometry or immunofluorescence analysis

## Tumor Dissociation

Explanted tumors were cut in small pieces with a scissor; pieces were covered with a digestive solution composed of collagenase IV (1 mg/ml) hyaluronidase (0,1 mg/ml) and DNase (0,03 KU/ml) and incubated at 37°C; every 10 minutes tumors were mechanically processed using a 5 ml pipette. After 1 hour, cells were collected and washed twice in complete medium to remove all digestive solution.

## Cytokine Nitration

Recombinant mouse GM-CSF was purchased from Miltenyi Biotech (130-095-742). Cytokine nitration was performed by adding 1mM peroxynitrite (Millipore) to the recombinant protein at 37°C for 15 min in a final volume of 100ul PBS (Lonza). After incubation, the samples were extensively dialyzed in PBS for 5 hours using the Slide-A-Lyzer Dialysis cassette kit, 3,500 MWCO (Thermo Fisher Scientific). Finally, cytokines were collected and used for *in vitro* assays at 20 ng/ml.

## LC-MS/MS Analysis

Equal amounts of recombinant GM-CSF(1 μg) either untreated or treated with peroxynitrite or with degraded RNS were digested using sequencing-grade modified trypsin (Promega) with a protein/enzyme ratio of 50:1, as described in (18). Peptides were injected into a 10 cm pico-frit capillary column (75 μm, I.D. New Objective) packed in house with $C_{18}$ material (ReproSil, 300A°, 3 μm, Dr. Maisch GmbH) using a Ultimate 3000 HPLC system (Dionex – Thermo Fisher Sicentific) and separated using a linear gradient of acetonitrile/0.1% formic acid from 3% to 40% in 15 min at a flow rate of 250 nl/min. Eluted peptides were analyzed using a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific) coupled online with the LC system through a nano-spray ion source (Thermo Fisher Scientific). The MS system operated in a data dependent mode, by acquiring a full MS scan at high resolution in the Orbitrap analyzer, followed by the MS/MS scans in the linear Ion Trap of the 10 most intense ions. Data were searched using Mascot Search Engine (Matrix Science) against the SwissProt database (Version 2013_04, 539829 sequences; 191670831 residues). Precursor and fragment ion tolerance were set to 10 ppm, and 0.6 Da respectively. An error tolerant search was done to highlight possible modifications and nitration of Tryptophan 30 (W30) was identified as one of the main post-translational modifications, which was present at high concentration only in the sample treated with peroxynitrite. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium *via* the PRIDE (19) partner repository with the dataset identifier PXD027049.

## Bone-Marrow Derived MDSC

C57BL/6 mice were sacrificed, tibias and femurs were removed to flush the bone-marrow under sterile conditions. Red cells were lysed with ACK Lysing Buffer (Lonza). To obtain MDSCs $2x10^6$ cells were plated into 6-well plate in medium supplemented with 20 ng/ml of GM-CSF (Miltenyi) native or nitrated by peroxynitrite (Millipore) in combination with 20 ng/ml of IL-6 and cultured for 5 days at 37°C in 5%$CO_2$.

## Cytofluorimetric Analysis

Cells derived from *in vitro* bone marrow differentiation or cells isolated from tumor-bearing mice, were blocked with FcR binding sites and stained with the following antibodies: anti-CD11b (clone M1/70), anti-Ly6G (clone 1A8), anti-F480 (clone CI:A3-1), anti-Ly6C (clone HK1.4), anti-pStat3, anti-p-Stat5, anti-p-Erk, anti-CD45.1 (cloneA20). Aqua Live/Dead®dye (Invitrogen) was used to analyze cell viability. Intracellular staining was performed using BD Cytofix/Cytoperm Kit in association with BD Phosflow Perm Buffer III. Flow data were acquired with FACSAriaII and analysed with FlowJo software (Tree Star, Inc.).

## Immunofluorescence

Mouse tumors were collected and fixed in 4% paraformaldehyde, cryoprotected in 30% sucrose and frozen in OCT. The samples were cut with cryostat (7μm), incubated with blocking solution (10% FBS, 3% BSA) 2 hours at room temperature. The following primary antibodies were used: anti-nitrotyrosine (Millipore), anti-nitrotryptophan (JaICA), anti-F480 (Abcam), anti-CD31 (R&D Systems). The appropriate secondary antibodies were used. For quantitative analysis, 10 different and non-contiguous regions of interest (ROIs) were randomly selected, and percentage of positive areas was obtained for each marker. Images were acquired with a Leica TCS SP5 confocal microscope. Images and colocalization indexes were analyzed by ImageJ Software.

## Immunohistochemistry

Human colorectal specimens (n=6) were obtained from the Padua Hospital in accordance with the ethical standards. This study was examined and approved by the Ethics Committee of the local Health and Social Services (Azienda Ospedaliera, Padova) in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. Specimens were frozen in OCT, cut with cryostat (7μm) and analysed with doublestain IHC Kit (Abcam) according to manufacturer's instructions. For quantitative analysis 10 different and non-contiguous regions of interest (ROIs) were randomly selected, and percentage of positive areas was obtained for each marker. Images were acquired by Olympus IX81and analyzed by ImageJ Software.

## RNA Purification and Real-Time PCR Analysis

To confirm the presence of GM-CSF encoding sequences, RNA extraction was performed using RNeasy Mini Kit (QIAGEN). cDNA was obtained with SuperScript II reverse transcriptase and poly dT primers (Invitrogen). PCR was performed in a volume reaction of 10ul containing TaqMan Universal PCR Master Mix (Applied Biosystem) and 20 ng cDNA with the following couple of primers (5' – 3' etc). We used Applied Biosystems 7900HT-Fast Real-time for PCR reaction and fluorescence detection. All measures were performed in duplicates and were analyzed by the ΔΔCt relative quantification method: arithmetic means of the cycle threshold (Ct) values were calculated and target gene mean Ct values were normalized to the respective endogenous control (*Gapdh*). The values obtained were converted by the formula 2 (−ΔΔCt) to be expressed as fold changes in regulation compared with the reference sample.

## Mixed Lymphocyte Peptide Culture and Proliferation Assay

MLPC cultures were prepared by mixing γ-irradiated C57BL/6 splenocytes with OT-I/CD45.1 splenocytes in order to obtain 1% OVA-specific T lymphocytes in the final culture. OVA-specific lymphocytes were previously CFSE-labeled (carboxyfluorescein succinimidyl ester, Cell Trace Kit, Invitrogen Molecular Probe) according to manufacturer's instructions for the proliferation assay. A total of $0.6x10^6$ mixed cells were plated in flat-bottom 96-well plates and stimulated for 3 days with 1 μg/ml of OVA peptide ($OVA_{257-264}$, SIINFEKL). Where required, MDSCs (derived from immunomagnetic sorting) were added at decreasing percentages (12% and 6%). T Cell proliferation was evaluated by FACS and data analyzed by FlowJo software (Tree Star, Inc.) using the *FlowJo's Proliferation* Tool by tracking cell generation according to software indications.

## ELISA

The GM-CSF concentrations in cell culture supernatants and in serum from mice were evaluated using mouse GM-CSF Quantikine ELISA kit (MGM00 R&D System, Minneapolis, MN) according with manufacturer's instructions.

## Western Blot

Total protein extract was obtained with RIPA buffer. Protein extracts were separated by 4-12% Bold NuPage (ThermoScientific) and transferred onto PVDF membranes (BioRad). After blocking with 3% albumin (Sigma-Aldrich) and primary antibody incubation phospho-STAT5, phospho-Erk1/2, Erk1/2 -Cell Signaling- and STAT5 -Santa Cruz- 1:1000), the membranes were incubated with an anti-rabbit peroxidase-conjugated secondary antibody (GE healthcare). Chemiluminescence was obtained by the ICL Substrate (GE healthcare), and images were obtained with an imaging ImageQuant LAS 500 (GE healthcare).

## Statistical Analysis

Statistical comparison between two groups was carried out using the Student's T test or Mann Whitney test. For multiple comparisons, One-way Anova test, followed by a Tukey post-hoc test was performed. Results with a p-value ≤ 0.05 were considered as significant. In figures, asterisks were used as follows: *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001. Analyses were performed using Graph Pad Prism (version 7).

# RESULTS

## Cytokine Nitration Affects Myeloid Cell Commitment by Activating Multiple Signaling Pathways

GM-CSF is commonly up regulated in multiple human tumors where it exerts a recognized tumor-promoting action (20). Nonetheless, evidence indicates a supportive role for this cytokine in fostering anti-tumor immunity (21). In a previous report, we revealed that the nitration of the CCL2 chemokine, caused by the oxidative overload within the tumor mass, impacted on immune cell trafficking by selectively affecting anti-tumoral T cell infiltration (18). Here, we reasoned that post-translational modification (PTM) could be responsible for the antithetical course of action of GM-CSF in tumors. To test our hypothesis, we at first assessed whether GM-CSF represents a ROS/RNS target by timely exposing the recombinant (human and mouse) GM-CSF to commercial peroxynitrite ($ONOO^-$); the presence of reproducible modifications as nitration/nitrosylation and/or oxydation was evaluated by tandem mass spectrometry coupled to liquid chromatography (LC-MS/MS). Our results indicated that a single Tryptophan residue, in the SPITVTRPW*K peptide was stably nitrated, as demonstrated by a +45Da shift in the tryptophan residue (where * represents nitration, +45 Da) upon peroxynitrite treatment (**Supplementary Figure 1A**). The accumulation of myeloid immunosuppressive cells (MDSCs) at the tumor site correlates with bad prognosis in different type of cancers (22) and it has been well-established that GM-CSF acts as driver for MDSC differentiation in tumors (12). To investigate the effect of GM-CSF nitration on MDSC commitment and functions, we generated *in vitro* fully competent MDSCs from bone marrow precursors (BM-MDSCs) by adding GM-CSF and IL-6 cytokines as previously published (11). In particular, we used $ONOO^-$treated GM-CSF (called hereafter N-GM-CSF) or GM-CSF treated with

degraded $ONOO^-$ (control) alone, or in combination with IL-6. After 5 days of culture, we phenotypically profiled harvested culture cells by multiparametric flow-cytometry (FACS). Interestingly, FACS analysis revealed a higher expansion of the PMN-MDSC subset in cells differentiated in presence of N-GM-CSF, alone or in combination with IL-6, in comparison to cells differentiated with the unmodified cytokine. No significant differences were observed for the Mo-MDSC subset. Moreover, N-GM-CSF exposure induced an increment of macrophages ($Ly6C^{low/-}$ $Ly6G^-$ $F4/80^+$) and a concomitant reduction of $Ly6C^{low/-}Ly6G^-$ cells (**Figure 1A** and **Supplementary Figure 1B**). Further, we evaluated the effect of N-GM-CSF on the immunosuppressive functions of BM-MDSCs. OVA specific CFSE-labeled T lymphocytes were stimulated to proliferate by adding the specific OVA-peptide to the culture, in the presence or not of different concentrations of BM-MDSCs, previously differentiated with either N-GM-CSF+IL-6 or GM-CSF+IL-6 cytokines. We observed that MDSCs obtained from the N-GM-CSF+IL-6 cultures were more efficient in inhibiting $CD8^+$ T lymphocyte proliferation compared to the GM-CSF/IL-6 cultures (**Figure 1B**). Our results indicated that the biological activity of GM-CSF is significantly altered by protein nitration.

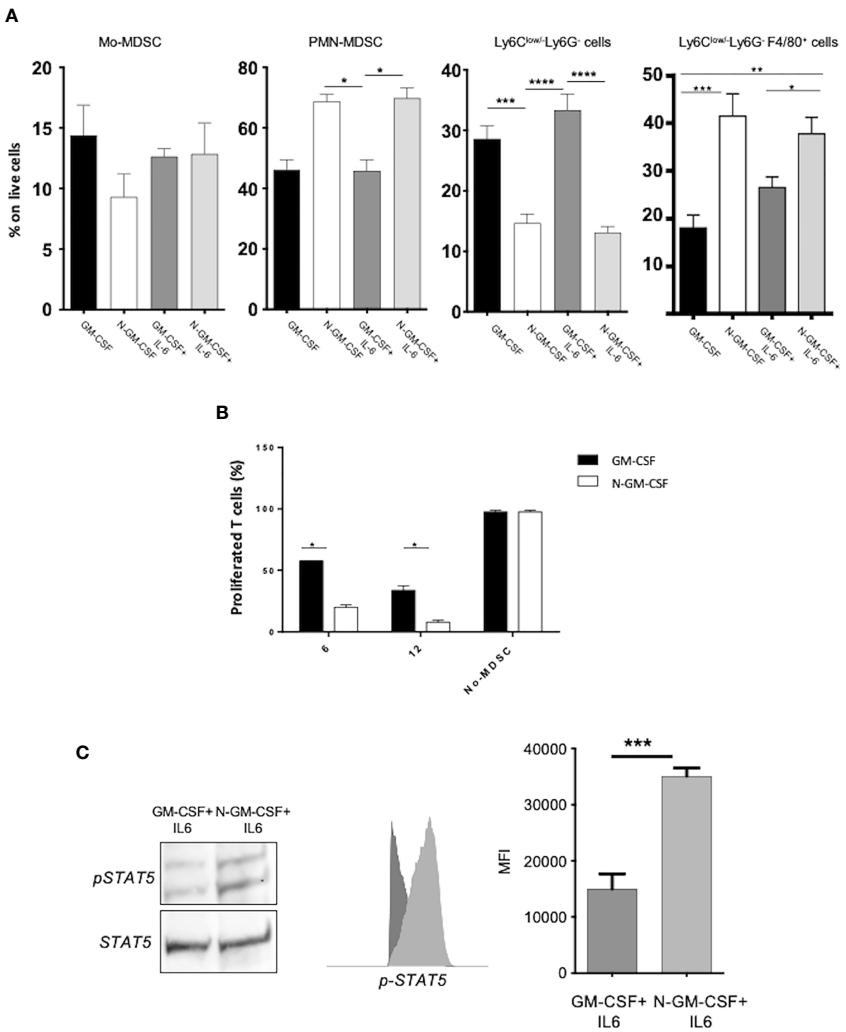


**FIGURE 1** | GM-CSF nitration boosts myeloid cell commitment and activates immunosuppressive-related signaling pathways. **(A)** Bone-marrow progenitors were cultured in the presence of either untreated GM-CSF or with peroxynitrite-treated GM-CSF (N-GM-CSF) alone or in combination with recombinant IL-6. The Bar graph represents the percentage of cells for each subset on $CD11b^+$ myeloid cells after 4 days of culture. Data represent means ± s.e.m from 6 independent experiments; *p ≤ 0.05; **p ≤ 0.01: ***p ≤ 0.001) **(B)** Different percentages (6-12%) of BM-MDSCs obtained after 5 days of culture with either GM-CSF + IL-6 or N-GM-CSF + IL-6 were tested for their ability to suppress the proliferation of OVA-specific, OT-I $CD8^+$ T cells stimulated by the SIINFEKL peptide in culture. Bar graph represents the percentage of proliferating T cells. OT-I $CD8^+$ T cells stimulated with peptide in the absence of BM-MDSC were used as control. **(C)** Representative WB and FACS analysis of the phosphorylation level (expressed as MFI) of STAT5 protein in BM-MDSCs differentiated by GM-CSF or N-GM-CSF or in combination with IL-6. Data represent means ± s.e.m from 4 independent experiments. Significance was determined by one-way Anova (Tukey test for multiple comparisons; *p ≤ 0.05; **p ≤ 0.01: ***p ≤ 0.001; ****p < 0.0001).

Indeed, N-GM-CSF sustains *in vitro* the expansion of myeloid cell subsets endowed with higher immunosuppressive activity as compared to the unmodified cytokine.

We next sought to investigate the molecular pathways triggered in myeloid cells upon N-GM-CSF stimulation. It is known that GM-CSF interaction with its receptor, the GM-CSFR, activates the Erk1/2 kinases and downstream selected transcriptional factors including among others, STAT5, which in turn regulates cell proliferation, differentiation and survival (23). In particular, STAT5 phosphorylation has been linked to T cell suppression by promoting apoptosis and Erk1/2 kinases was associated with myeloid precursors differentiation (4, 24). We found that N-GM-CSF, in combination with IL-6, induced a significant increment in the phosphorylation level of STAT5 and Erk1/2 in N-GM-CSF-derived MDSCs, as compared to the unmodified cytokine, (**Figure 1C** and **Supplementary Figure 2**). Collectively, our data suggested that the nitration of GM-CSF impinges on myeloid cell commitment and immunosuppressive activity possibly boosting the STAT5 and MAPK pathways in differentiating BM-precursors.

## N-GM-CSF Affects Myeloid Cell Commitment and Activity in Tumor-Bearing Hosts

To specifically address the impact of RNS-modified GM-CSF in tumor progression, we genetically modified the murine fibrosarcoma cell line MCA-203, that did not secrete *per se* detectable levels of GM-CSF both *in vitro* and *in vivo*. Specifically, we generated tumor-cell clones stably expressing either a plasmid encoding the full sequence of the murine GM-CSF (named GM) or a full-mutant sequence (named W30L), where we introduced a point mutation at the site of protein nitration. In more detail, we substituted the RNS-targeted amino acid residue (tryptophan, W) with a no-targetable one, by a site-specific mutagenesis approach. We changed the codon encoding W in position 30 of murine GM-CSF with the one for leucine (L). This substitution was driven by a bioinformatic analysis where different homologues and orthologues protein sequences where aligned. Interestingly, our analysis indicated that this position is well conserved, with very few cases where other amino acids are encoded. Moreover, the codon for leucine has the highest substitution score according to two independent substitution matrix (PAM250 and Blosum) and is not known to be sensitive to nitration/nitrosylation such as other aromatic residues or polar residues (25) (**Supplementary Figures 3A, B**). Computational modelling analysis of murine GM-CSF structure confirmed that the tryptophan in position 30 (W30) is exposed to the solvent and is a good candidate for protein nitration. This analysis also highlighted the stability of the W30L mutant (**Supplementary Figure 3C**). By this strategy, we obtained multiple MCA-203 clones expressing either the GM or the W30L plasmids. Among them, we selected a pair of clones, hereafter named GM and a W30L, which displayed, *in vitro*, the same ability to proliferate (data not shown) and that were able to secrete comparable amount of GM -CSF (**Supplementary Figure 4A**). Supernatants from GM and W30L clone cultures were tested for their ability to differentiate *in vitro* MDCSs from BM precursors. Recombinant GM-CSF and IL-6 were used as controls. We did not observe differences in the percentage and morphology of MDSC subsets (Mo-MDSC, PMN-MDSC and $Ly6C^{low/-}$ $Ly6G^{-}$) obtained from BM cell cultures upon the stimulation with either MCA203-GM or MCA203-W30L secretome. However as expected, cell supernatants were less effective than the recombinant cytokines in differentiating myeloid cell populations (**Supplementary Figures 4B, C**).

To dissect the impact of GM-CSF nitration *in vivo*, we challenged immunocompetent C57BL/6 mice with either MCA203-GM or MCA203-W30L tumor cells. We did not observe significant differences in the tumor growth between the two engrafted clones (**Figure 2A**). Moreover, we detected comparable amounts of circulating GM-CSF in the serum of mice injected with the two different tumor clones (**Figure 2B**). Three weeks later the MCA-203 challenging, we analyzed myeloid cell accumulation at the tumor site. We did not find any significant variations in the total percentage of $CD11b^{+}$ cells (around 30% of intra-tumoral MDSCs on total tumor cells) but we observed a significant reduction in the percentage of PMN-MDSCs ($Ly6G^{+}$) and macrophages ($Ly6C^{-}/Ly6G^{-}/F4/80^{+}$) in mice MCA203-W30L-bearing mice compared to the MCA203-GM controls (**Figure 2C** and **Supplementary Figure 5A**). These data suggested that the nitration of the GM-CSF amplifies the recruitment and commitment of immunosuppressive myeloid cells in tumors.

We also evaluate the level of N-W at the tumor core by confocal microscopy and, as expected we found a significant reduction in tryptophan nitration in W30L tumors compared to GM controls. At the same time, we detected a significant reduction in the nitro-tyrosine content in MCA203-W30L tumors suggesting that the nitration of GM-CSF locoregionally tuned the recruitment and expansion of cell subsets that can sustain the oxidative/nitrosative stress at the tumor core (**Figures 2D, E** and **Supplementary Figure 5B**). Evidence indicated that GM-CSF also promotes immune-independent tumor progression by directly supporting cancer cell survival and tumor angiogenesis (26). We observed a consistent decrease in the endothelial marker CD31 in W30L tumors compared to GM controls (**Figures 2D, E** and **Supplementary Figure 5B**), suggesting that GM-CSF nitration might also affect tumor angiogenesis.

The spleen plays a crucial role during tumor progression since it represents the elective site for the induction of tumor tolerance mediated by myeloid cells (27). Indeed, we moved to characterize myeloid cell landscape in the spleen of either MCA-GM or W30L-GM tumor-bearing mice. We found a significant increase of immature monocytes ($Ly6C^{mid}$) and macrophages in the spleens of W30L tumor bearing mice as compared to the control (**Figure 3A**). To evaluate the functional phenotype of these myeloid cell populations, we isolated $CD11b^{+}$ cells from the spleens of either MCA203-W30L or MCA203-GM-bearing mice, and we assessed their immunosuppressive activity by co-culturing $CD11b^{+}$ with antigen-activated T lymphocytes. Remarkably, we observed that MDSCs from W30L tumors were less efficient in suppressing T cell proliferation in comparison to the GM controls (**Figure 3B**). Finally, as MDSC immunosuppressive potential in tumors mainly rely on the expression and activity of two key enzymes, Arginase 1

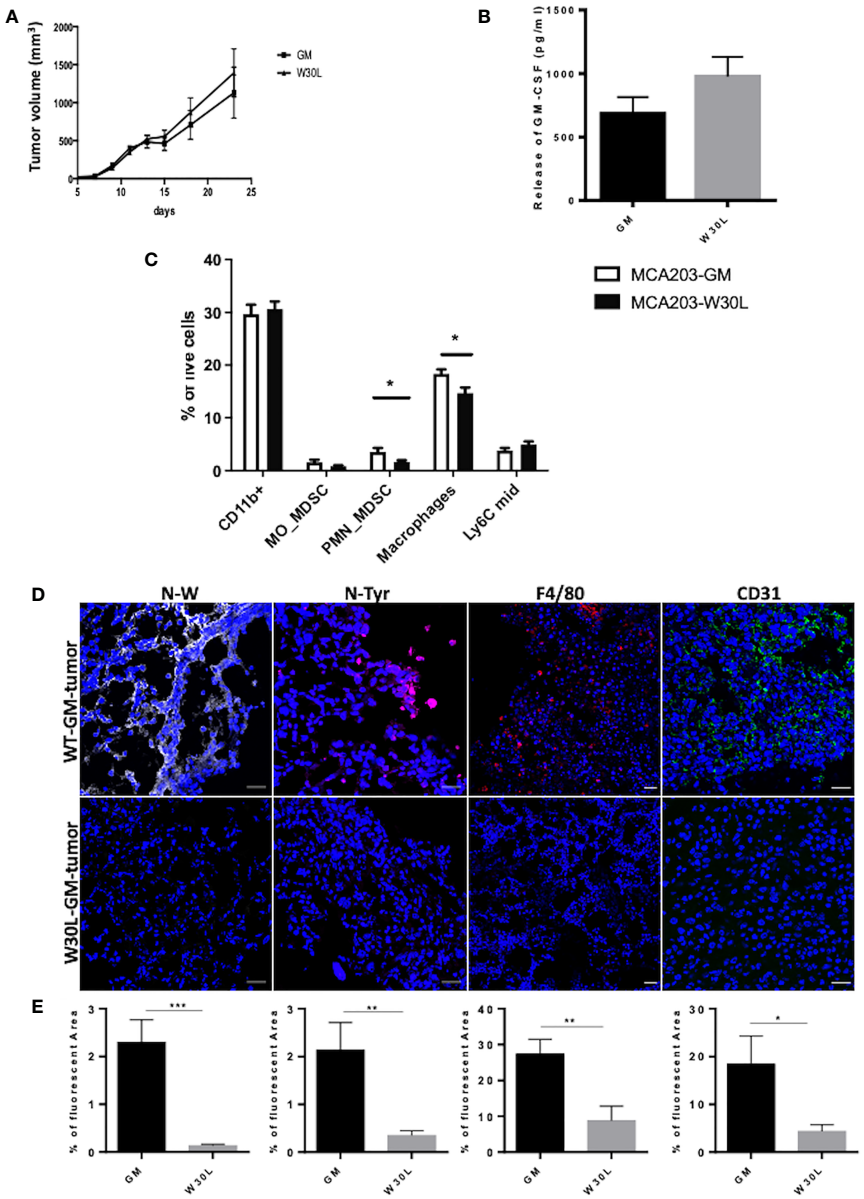


**FIGURE 2** | The nitration of GM-CSF impacts on myeloid cell frequency within the tumor microenvironment **(A)** C57BL6 mice were s.c challenged with either MCA203-GM or MCA203-W30L tumor cell clones. Tumor volume was measured at the indicated time-points; **(B)** At day 21 after tumor injection the amount of GM-CSF in serum of mice was quantified by ELISA. **(C)** Frequency of myeloid subsets on live cells in tumor tissues. Error bars represent means ± s.e.m; data are representative of 3 independent experiments unpaired Student t test analysis (*$p \leq 0.05$) **(D)** Representative images of IF of tissue slices from either MCA203-GM or MCA203-W30L tumors. Tissues were stained for nitro-tryptophan (grey), nitro-tyrosine (magenta) F4/80 (red) or CD31 (green). **(E)** Quantification of fluorescence (positive reactive areas) from nitro-tryptophan, nitro-tyrosine, F4/80, CD31 staining. Scale bar, 25 μm. Data represent means ± s.e.m from 3 independent experiments; unpaired Student T-test *$p \leq 0.05$; **$p \leq 0.01$: ***$p \leq 0.001$.

(ARG1) and Nitric Oxide Synthase 2 (NOS2), we checked by Real time-PCR the transcription level of these enzymes in $CD11b^+$ cells isolated from the spleens of tumor-bearing mice. Importantly, we found that myeloid cells from MCA203-W30L tumor-bearing mice expressed lower level of both ARG1 and NOS2 enzymes as compared to the control (**Figure 3C**), that could potentially explain their reduced suppressive activity. Collectively, our findings indicated that the nitration of GM-CSF deeply conditions tumor immune contexture and more, it boosts the systemic expansion and activity of MDSC in tumor bearing hosts.

## Tryptophan Nitration in Colorectal Cancer

The expression of GM-CSF in human colorectal primary tumors has been extensively documented. Interestingly, elevated levels of

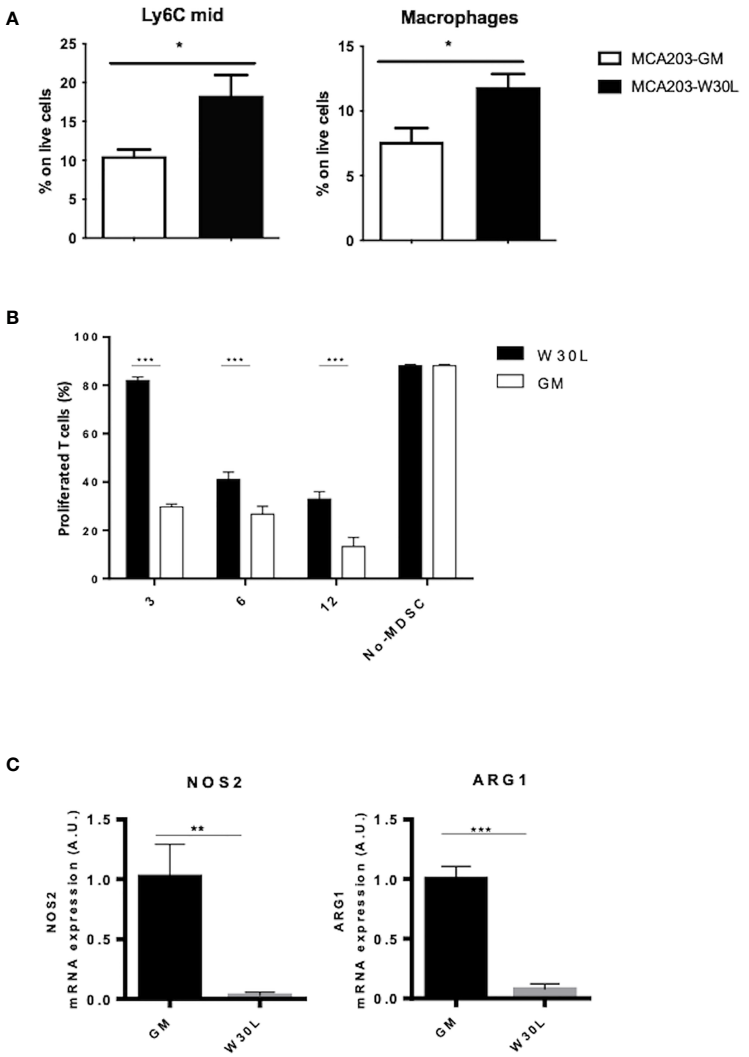


**FIGURE 3** | The nitration of GM-CSF affects the differentiation and activity of myeloid cells in tumor-bearing hosts. **(A)** C57BL6 mice were s.c. challenged with either MCA203-GM or MCA203-W30L tumor clones. At day 21, after tumor challenge mice were sacrificed and myeloid cell subpopulation were quantified by FACS. **(B)** $CD11b^+$ cells magnetically-purified from the spleen of either MCA203-GM or MCA203-W30L tumor-bearing mice were co-cultured with OVA-specific, CFSE-labeled, $CD8^+$ T lymphocytes in the presence of OVA peptide. Bar graph shows the percent of proliferating T cells. Lymphocytes stimulated with OVA peptide in absence of myeloid cells (no-MDSC) were used as controls. **(C)** NOS2 and ARG1 mRNA expression was evaluated by RT-PCR Data represent means ± s.e.m from 3 independent experiments; unpaired Student T-test *$p \leq 0.05$; **$p \leq 0.01$: ***$p \leq 0.001$.

soluble GM-CSF have been recognized in the serum of colorectal cancer patients, suggesting that GM-CSF may be an independent prognostic factor (28). However, the role of cytokine in colorectal cancer still needs to be defined being GM-CSF endorsed with either tumor -suppressing or tumor-promoting activity in human patients (20, 29). Indeed, we assessed the expression of GM-CSF in colorectal primary tumor specimens and we observed that GM-CSF is more abundant at the core of the lesion in comparison to the surrounding non tumoral epithelium (NT-tissue) (**Figures 4A, B** and **Supplementary Figures 6SA, B**). We next evaluated the presence, the distribution and the abundance of nitrated-Tryptophan (N-W) in the same tumor specimens by using an antibody specifically recognizing nitro-tryptophan residues. Our analysis revealed a significant increment of N-W at the neoplastic foci compared to the surrounding not-transformed tissue (**Figures 4A, B** and **Supplementary Figures 6SA, B**). The level of the oxidative/nitrosative level in the very same tissues was also confirmed by the nitro-Tyrosine marker (**Supplementary Figures 7A, B**). Finally, by using two co-localization indexes we revealed that GM-CSF co-localized with N-W at higher extent in tumors compared to the control surrounding tissues (**Figure 4C**).

Overall, our data indicated that a high rate of oxidative/nitrosative stress characterized the core of the human tumor lesion where the level of GM-CSF is elevated as compared to the normal epithelium.

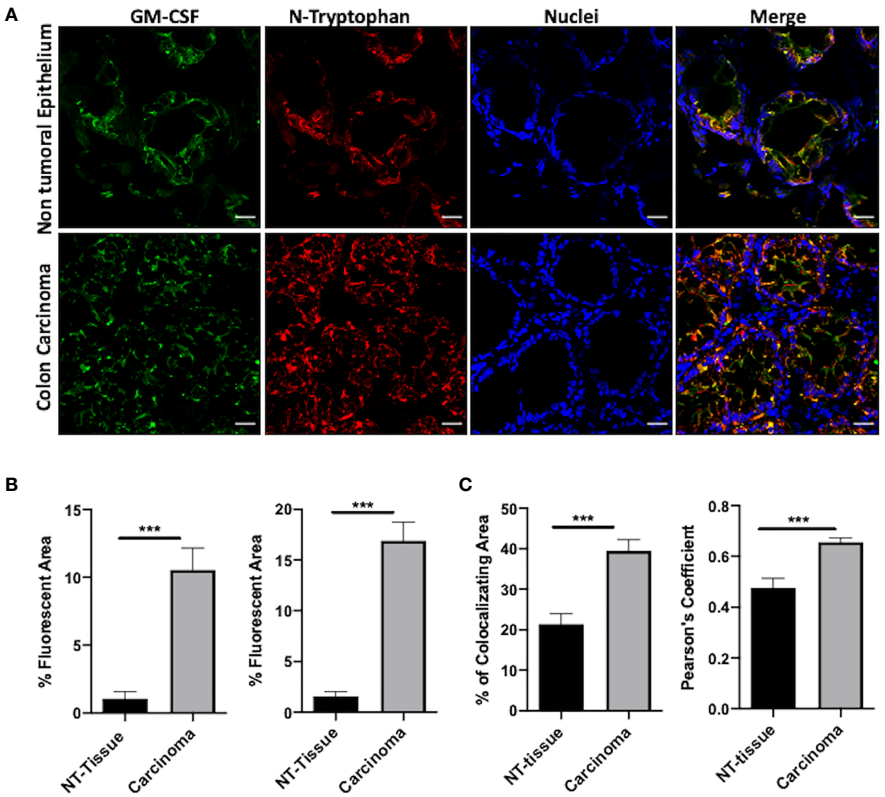


**FIGURE 4** | N-GM-CSF in human colon carcinomas. **(A)** IF images of a specific area in human colon carcinoma samples. Blue: nuclei; red: nitro-tryptophan, green: GM-CSF; yellow:merge; **(B)** Quantification of fluorescence (positive reactive areas) for GM-CSF and nitro-tryptophan staining. Scale bar, 25 μm. **(C)** Percentage of reactive areas where nitro-tryptophan and GM-CSF co-localized in either carcinomas or NT tissues (upper graph). Pearson Coefficient of co-localization for nitro-tryptophan and GM-CSF in either carcinomas or NT-tissues (bottom graph). Data are representative of n = 6 patient biopsies, unpaired Student T-test analysis (***$p < 0.001$).

## DISCUSSION

Reactive oxygen species, including RNS that cause protein nitration/nitrosylation, are constantly generated in inflamed and transformed tissues as indicated by the high expression level of nitrosative stress markers in different human cancers (30). Our group previously discovered that the nitration of the inflammatory chemokine CCL2 limited T cell access into the tumor lesion by altering its chemoattractant properties (18). Nonetheless, the network of possible RNS-induced protein modifications within tumor specimens and the impact of such post-translational changes on the biology of the affected proteins can be expanded. In this study, we focused on a key cytokine - GM-CSF- that is abundantly secreted by several human tumors and plays a dual and still unclear role in cancer biology. Indeed, GM-CSF can act as a powerful stimulator of anti-tumor responses by favoring the recruitment and activity of NK cells, granulocytes, macrophages and antigen presenting cells in tumor-bearing hosts (31). Because of this rationale, GM-CSF has been proposed and employed as adjuvants in cancer immunotherapies. Clinical trials worldwide so far enrolled GM-CSF secreting tumor vaccines to treat patients with metastatic melanoma (31) prostate (32) and metastatic non-small-cell-lung cancer (33). Nevertheless, the clinical exploitation of this cytokine has showed conflicting results depending on cancer type and staging. Indeed in contrast with previous observations, it has been reported that high doses of GM-CSF can sustain cancer-related immunosuppression by increasing the number of immature myeloid cells (MDSCs) that actively suppresses anti-tumor T cell immunity (34). Later, *in vivo* evidence mechanistically connected GM-CSF to the intratumoral accumulation of highly immunosuppressive Gr-1$^{+}$CD11b$^{+}$ myeloid cells in a spontaneous pancreatic ductal adenocarcinoma (PDAC) model (12).

Taken together, all these findings indicated that the biology of the GM-CSF in tumors needs to be further expanded in order to reconcile the dual nature of this cytokine in cancer. Our study shed light on GM-CSF activity in tumors by indicating that this cytokine acts as a pliable element in cancer hosts. Indeed, we found that GM-CSF represents a new target for RNS in tumors. Furthermore, we investigated the functional significance of RNS-induced PTM for the biology of this cytokine. Specifically, by performing MS/MS analysis on *in vitro* peroxynitrite-treated protein we singled-out a specific aromatic residue, a tryptophan (W) which can be stably and consistently nitrated by RNS. The propensity of such residues to be modified by RNS

was strengthened by a bioinformatic approach (homology modeling of the human GM-CSF) that clearly confirmed the accessibility of such residues for the interaction with radical species. Importantly, we found that RNS-modified GM-CSF can act as an amplifier of cancer-related immunosuppression by enhancing the generation and activity of myeloid suppressive cell subsets.

A key process occurring during tumor progression is the pathologic expansion and activation of distinct myeloid cell subsets that suppress adaptive immunity (35), collectively named MDSCs. Despite the considerable literature on MDCS classification, clinical studies have reported a positive correlation between infiltrating-myeloid cell percentage and stage progression in different human tumors as melanoma, advanced renal cancer (36), breast, bladder (37) and colon rectal carcinomas (38).

For this reason, MDSCs are now recognized as a worthy diagnostic marker and importantly as a key target for cancer immunotherapy. Our data showed that N-GM CSF promotes the expansion of PMN-MDSCs and macrophages, which represent highly immunosuppressive subsets.

The induction of MDSC phenotype and activity relies on multiple signaling pathways downstream cytokine stimulation, including GM-CSF. The canonical pathway triggered by the GM-CSF binding to its receptor involves primarily MAP kinases and STAT family members. In particular, STAT5 phosphorylation sustains the maturation of dendritic cells and it is commonly associated with pro-inflammatory responses. The duality in GM-CSF molecular signature is consistent with the complexity of cytokine-driven responses and it could be explained on the basis of GM-CSF dosage, signaling amplitude, and timing. Remarkably, we found that N-GM-CSF significantly amplifies the molecular signaling leading to MDSC expansion and activity compared to the native, unmodified GM-CSF. Our data indicated that N-GM-CSF can boost GM-CSF signaling through the STAT5 pathway. In tumor tissues where RNS are present at a very high extent (30) the post-translationally-modified cytokine competes with the native one for receptor binding. The selective ablation of N-GM-CSF so far could revert this balance by lowering the activation of immunosuppressive pathways thus promoting anti-tumor (pro-inflammatory) responses in tumor-bearing hosts. Importantly, our *in vivo* data confirmed that the nitration of GM-CSF represents a founding element for MDCS expansion and recruitment in tumor tissues. To deal with this issue, we engineered a fibrosarcoma cell line by introducing a single point-mutation in GM-CSF sequence, generating a tumor model that stably secretes a GM-CSF mutant which is insensitive to RNS-induced PTM. Indeed, the substitution of the site of RNS attack –a tryptophan- redraws the immune landscape at the tumor site. Tumors secreting the genetically-modified GM-CSF insensitive to RNS action (the W30L mutant) display a significant decrease in the number of both PMN-MDSC and macrophages (TAM) at the primary neoplastic foci. This occurs with comparable serum levels of the "native" *versus* mutated GM-CSF. For ethical reason we ceased tumor observation at day 21. We found no differences in the overall growth of tumor masses between tumor cell lines secreting either WT or W30L GM-CSF at this time-point. Nonetheless, the volume of W30L tumors was slightly decreasing from day 15, reasonably suggesting that the decreased recruitment of immunosuppressive subsets at the primary site could affect tumor growth on a longer timeframe. Consistently, the rate of nitrosivative stress was significantly decreased in W30L tumors compared to control as indicated by the reduced expression of both nitro-tyrosine and nitro-tryptophan markers.

The expansion and recruitment of PMN-MDCSs have been clearly connected to the promotion of cancer progression in different tumor models (39, 40) and human carcinomas (41). PMN-MDSCs can act in tumor bearing hosts by exploiting multiple mechanisms as the promotion of cancer-related angiogenesis (42) and the production of highly reactive species as peroxynitrite, $O^{2-}$, and $H_2O_2$ (43). We also observed a trend in the decreasing of Mo-MDSC in W30L mice. At the tumor site, Mo-MDSC are rapidly committed to TAMs that historically exert a high ability to suppress T cell responses, in particular by producing nitric oxide and immune suppressive cytokines and expressing Arg1 (44).

Our data indicated that the nitration of GM-CSF represents a determinant element for the recruitment and expansion of both PMN-MDSC and TAM thus representing a promising target for immune-based therapy.

As largely reviewed (45), MDSCs originated from common BM precursors. Nonetheless, BM-MDCSs are poorly suppressive cells that need to exit this privileged site to exert their immunosuppressive activity. In line with this, MDCSs populating primary tumors and other sites as liver, blood and spleen can efficiently block T lymphocyte responses (40). In particular, the spleen represents an elective site for tumor-induced tolerance and remarkably splenectomy in tumor-bearing mice can reactivate anti-tumor immunity (27).

Of note, MDSCs isolated from the spleen of W30L GM-CSF tumor-bearing mice display a lower immunosuppressive activity compared to control WT tumors. Two enzymes participating to arginine-metabolism NOS2 and ARG1 are up-regulated in tumor specimens where act as key mediators for T cell suppressive mechanisms (7, 30). Importantly, the concomitant blockage of ARG1/NOS2 expression and activity harness MDSC ability to suppress T cell immunity (18). We found that NOS2 and ARG1 expression are significantly decreased in MDSCs isolated from W30L spleens, accounting for their reduced immunosuppressive activity in comparison to control cells. Indeed, our data confirmed that the nitration of GM-CSF nourishes the expansion, recruitment and activity of highly immunosuppressive myeloid cells both at the tumor primary site and systemically.

Along with the immune contexture, tumor vasculature plays a crucial role in dictating cancer progression and spreading. Indeed, growing tumors push the formation of new blood vessels to face their need of nutrients and oxygen supply. It is well established that oxidative/nitrosative stress promotes the generation of new vasculature sprouts within the tumor environment (46) representing so far a fascinating target for cancer therapy. In line with this, we observed that the reduction of the nitration rate in W30L tumor tissues goes with a significant decrease of the endothelial cell marker CD31 in the

same specimens, thus suggesting a possible impairment of tumor angiogenesis in this context. The cross-talk between endothelial and immune cells is a relevant but still undefined issue in cancer biology. Indeed, angiogenic factors as VEGF-A and IL-8 contribute to the recruitment and expansion of distinctive immunosuppressive cell subsets as MDSCs and TAMs within the tumor environment (47). Mutually, MDSCs sustain tumor angiogenesis by the secretion of pro-angiogenic factors such as MMPs, VEGF and basic fibroblast growth factor (bFGF), or importantly by their direct embodiment into tumor-endothelia (48).

Cancer immunotherapy currently represents the most effective option for the treatment of patients with fatal cancers. A desirable achievement for immune-based treatments against cancer would be the definition of therapies that selectively harness anti-tumor immunity and overcome tumor immunosuppressive circuits. When we analyzed human colorectal specimens, we detected the presence of nitrated-W that colocalized with GM-CSF expression.

Although we are aware that colocalization approach does not provide clear-cut evidence for the presence of nitrated-GM-CSF within the tumor core, our findings suggested that the specific targeting of the nitrated-GM-CSF might represent an effective strategy to inhibit the tolerogenic activity of the modified protein in tumors without affecting the immunostimulatory properties of the native-unmodified cytokine.

Although further investigations are needed, our pre-clinical study pointed out nitrated- GM-CSF as a novel potential target for anti-cancer immune-based approaches.

## DATA AVAILABILITY STATEMENT

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium *via* the PRIDE (19) partner repository with the dataset identifier PXD027049.

## ETHICS STATEMENT

This study was examined and approved by the Ethics Committee of the local Health and Social Services (Azienda Ospedaliera, Padova,n.57841, 3.12.2013) in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the institutional protocols for animal care and approved by "Ministero della Salute". All experiments followed committee guidelines CEASA 15/3/2012.

## AUTHOR CONTRIBUTIONS

Conceptualization, BM and IM. Investigation BC, AA, CC, RS-R, AP, GIT, GA, FZ, CM, and LA. Writing- original draft, BM and IM. Critical data discussion, VB and AV. Funding acquisition, BM. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.718098/full#supplementary-material

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# Oxidative and Non-Oxidative Antimicrobial Activities of the Granzymes

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Cell-mediated cytotoxicity is an essential immune defense mechanism to fight against viral, bacterial or parasitic infections. Upon recognition of an infected target cell, killer lymphocytes form an immunological synapse to release the content of their cytotoxic granules. Cytotoxic granules of humans contain two membrane-disrupting proteins, perforin and granulysin, as well as a homologous family of five death-inducing serine proteases, the granzymes. The granzymes, after delivery into infected host cells by the membrane disrupting proteins, may contribute to the clearance of microbial pathogens through different mechanisms. The granzymes can induce host cell apoptosis, which deprives intracellular pathogens of their protective niche, therefore limiting their replication. However, many obligate intracellular pathogens have evolved mechanisms to inhibit programed cells death. To overcome these limitations, the granzymes can exert non-cytolytic antimicrobial activities by directly degrading microbial substrates or hijacked host proteins crucial for the replication or survival of the pathogens. The granzymes may also attack factors that mediate microbial virulence, therefore directly affecting their pathogenicity. Many mechanisms applied by the granzymes to eliminate infected cells and microbial pathogens rely on the induction of reactive oxygen species. These reactive oxygen species may be directly cytotoxic or enhance death programs triggered by the granzymes. Here, in the light of the latest advances, we review the antimicrobial activities of the granzymes in regards to their cytolytic and non-cytolytic activities to inhibit pathogen replication and invasion. We also discuss how reactive oxygen species contribute to the various antimicrobial mechanisms exerted by the granzymes.

**Keywords: granzymes, ROS - reactive oxygen species, caspases, antimicrobial defense, apoptosis, mitochondria**

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## INTRODUCTION

A key mechanism against intracellular pathogens, such as viruses, bacteria and parasites, is cell-mediated cytotoxicity exerted by killer lymphocytes of the innate and adaptive immune systems (1, 2). When these cytotoxic immune cells recognize cells infected with intracellular pathogens, they release their cytotoxic granule contents to eliminate the target cells and the intracellular pathogen.

Cytotoxicity is mediated by a group of highly homologous serine proteases, the granzymes (Gzms), that are localized in specialized lysosomes of the killer cells (3). The best-established biological role of the Gzms is the induction of programed cell death when these cytotoxic proteases are delivered into the target cells by perforin (PFN) (4–6). PNF is a pore-forming protein (7) that facilitates the uptake of other cytolytic granule components by enhancing endocytic uptake (8–10) and promoting endosomolysis to allow cytosolic release (11) (**Figure 1**). Lymphocytic cytotoxic granules of humans and some other mammals, but not rodents, contain another membrane-disrupting protein, granulysin (GNLY) (14). GNLY belongs to the saposin-like protein family (SAPLIP) that is characterized by a particular polypeptide motif and its affinity to a variety of lipids (15). GNLY was found to disrupt prokaryotic (but not eukaryotic (16, 17)) membranes and to kill bacteria, parasites and fungi *in vitro* (18).

While the Gzms-mediated induction of host cell apoptosis is well established, the mechanisms of intracellular pathogen elimination is far less clear constituting an emerging field in recent years. The most obvious mechanism is that host cell death deprives obligate intracellular pathogens of their protective niche. Host cell death plays a major role in the elimination of many viruses (19) and obligate intracellular bacteria, such as *Chlamydia* spp (20, 21). These kind of pathogens counteract the host cell death machinery with a variety of inhibitory mechanisms, such as prevention of cytochrome C release (22) or the upregulation and/or mimicry of host anti-apoptotic proteins (23–25).

In addition to the induction of cell death, a direct mechanism of antimicrobial activities by the Gzms was discovered in numerous studies, which is mediated by the proteolytic degradation of microbial proteins to activate microbial death pathways, hence, limiting their growth inside a host, independently of host cell death. In this review, we aim to dissect these mechanisms with a particular focus on oxidative *versus* non-oxidative killing pathways.

## THE GRANZYMES

The Gzms are a family of serine proteases firstly described by the team of Jürg Tschopp in 1986, who identified "granular enzymes" in the secretory granules of cytolytic lymphocytes (26). There are 5 human Gzms (A, B, H, K and M) and 10 mouse orthologues (A, B, C, D, E, F, G, K, M and N). The human Gzms are encoded from three different chromosomal loci: the chymase locus on chromosome 14 encodes for GzmB and GzmH, the met-ase locus on chromosome 19 for GzmM, and the tryptase locus on chromosome 5 harbors GzmA and GzmK (27). Although the human Gzms are highly homologous and share the catalytic triad ($His^{57}$, $Asp^{102}$ and $Ser^{195}$), there are remarkable differences in their primary substrate specificities (28). The tryptases GzmA and GzmK cleave substrates after Arg or Lys (29, 30), GzmB cleaves after Asp (31), GzmH cleaves after Tyr or Phe (32), and GzmM cleaves after Leu or Met (33).

It is noteworthy that the Gzms, in particular GzmB, are not only expressed and secreted by killer lymphocytes. They were also detected in various non-cytotoxic immune cells (34), non-immune cells (35) and cancer cells (36–38). Interestingly, the non-cytolytic activities of granzyme B also modulate the differentiation of lymphoid cells *via* the interference with NOTCH1 signaling (39) or with production of IL-17 (40). As PFN is exclusively expressed in activated killer lymphocytes (41), the Gzms released from the above listed cells will exhibit predominantly extracellular effects. Potential activities include remodeling of extracellular matrix (42), modulating inflammation (43–45), detachment-mediated cell death, anoikis (46), and – as reviewed below – exerting antimicrobial activity against invading pathogens in synergy with antimicrobial peptides (AMPs) or by targeting secreted microbial proteins.

## THE GRANZYMES IN CELL DEATH

Due to this diversity in the cleavage site specificity, all Gzms have their unique degradomes (47), resulting in the activation of widely differing death pathways in target cells after cytosolic delivery by PFN. The best characterized death pathways are those of GzmA (48) and GzmB (49). GzmB executes death in a caspase-dependent manner (50) (**Figure 1**). Once released into the cytosol of a target cell, GzmB can directly cleave several caspases (51, 52), including caspase 3 (53). This executioner caspase trigger the release of an active DNase (CAD), responsible for DNA fragmentation upon various apoptotic stimuli (54). Human GzmB also efficiently cleaves the pro-apoptotic protein Bid (55). Truncated Bid induces Bad/Bax-dependent mitochondrial outer membrane permeabilization, the release of cytochrome C, SMAC/DIABLO, and other proteins, such as Htra2/omi, ultimately leading to apoptosome formation and activation of caspase 9 (56–61).

GzmA induces a cell death harboring morphological features similar to apoptosis: chromatin condensation, nuclear fragmentation, membrane blebbing, mitochondrial swelling and loss of cristae. However, GzmA does not activate executioner caspases to kill the cell. Cytosolic delivery of GzmA triggers a complex cascade of events that includes the translocation of a protein complex known as SET from the ER to the nucleus, ultimately leading to the nuclear transfer of two nucleases (NM23-H1 and Trex1) and lethal DNA damage (62–64). These pro-apoptotic features and mechanisms of GzmA were essentially established in the laboratory of Judy Lieberman (65).

An important common death mechanism of GzmA and GzmB is nuclear uptake to attack several nuclear proteins, involved in structural integrity, DNA repair and RNA splicing (66–72).

For the residual, "orphan" Gzms, caspase-dependency to induce death or even if the induction of apoptosis is their major function is still not clear and needs further study (73, 74). However, there are multiple lines of evidence suggesting that GzmM, GzmH and GzmK, as well as the non-orphan GzmA,

have well defined proinflammatory and antimicrobial roles as further discussed below (75–78).

## OXIDATIVE CELL DEATH PATHWAYS DRIVEN BY THE GRANZYMES

A critical common feature of GzmA and GzmB death pathways is the mitochondrial uptake of these enzymes (**Figure 1**). Once in the mitochondria, the Gzms cleave four subunits of the respiratory chain complex 1 (NDUFS3, NDUFV1, NDUFS1 and NDUFS2). The disruption of the electron transport chain dramatically increases premature electron leakage, leading to the formation of reactive oxygen species (ROS), a decrease in mitochondrial respiration and the loss of cristae (79–81). Strikingly, caspase 3 also degrades a complex 1 subunit (NDUFS1) to induce ROS-dependent cell death (82–84). In challenge of the orthodox mitochondrial import biology, GzmA and GzmB (and potentially caspase 3), without containing a canonical mitochondrial import sequence, cross the outer mitochondrial membrane through SAM50 channels and the inner membrane through TIM22 in a mtHSP70-dependent manner (85). The resulting increased ROS generation facilitates the release of apoptogenic factors through Bax/Bak pores and drives the nuclear translocation of the SET complex to enhance GzmB and GzmA death pathways, respectively. At least one study suggests that there might be additional, extra-mitochondrial sources of ROS induced by GzmB, in particular *via* the activation of NADPH-oxidase (86).

## CYTOLYTIC ANTIMICROBIAL FUNCTIONS OF THE GRANZYMES

The induction of host cell death *via* the granule exocytosis pathway is an obvious effector mechanism used by killer lymphocytes to eliminate the host cells and obligate intracellular pathogens, such as viruses (87) and certain unicellular parasites (88) or bacteria (89). Suicidal death is an approved defense mechanism of cells infected with pathogens independently of a lymphocyte attack (90–92). Programed host cell death deprives the pathogens of their protective niche, minimizes the risk of dissemination as membrane integrity is initially preserved, and recruits and activates phagocytes to digest the remains (93). Therefore, it is not surprising that obligate

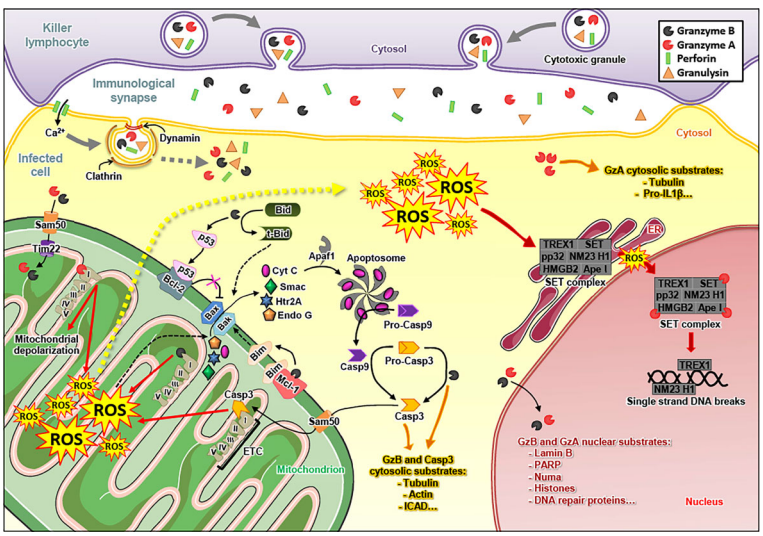


**FIGURE 1** | Granzyme A and Granzyme B induce apoptosis of infected cells. The killer lymphocyte releases the content of its granules in the immunological synapse, *i.e.* the immune effectors granzyme A (GzmA), granzyme B (GzmB), perforin (PFN) and granulysin (GNLY). The endocytosis of GzmA, GzmB, PFN and GNLY in the infected cell is mediated by a PFN-dependent calcium ($Ca^{2+}$) influx and relies on clathrin and dynamin. Once in the cytosol, GzmA and GzmB cleave various substrates. GzmB triggers the formation of Bax/Bak pores in the outer mitochondrial membrane by cleaving Bid (in truncated-Bid, t-Bid), Mcl-1 (which releases Bim) (12) and p53 (which inhibits Bcl2) (13). GzmB also directly cleaves and matures pro-caspase 3 (Pro-Casp3) into active caspase 3 (Casp3). GzmB and caspase 3 target similar substrates that induce apoptosis of the infected cell. GzmA and GzmB enter the mitochondria – *via* Sam50 and Tim22 – where they target subunits of the electron transport chain (ETC) complex I, leading to the production of reactive oxygen species (ROS). The ROS favor the release of apoptogenic factors through the Bax/Bak pores, such as cytochrome C (Cyt C), Smac, Htr2A and endonuclease G (Endo G) in the cytosol. Cyt C binds Apaf1 to form the apoptosome, which matures the pro-caspase 9 (pro-Casp9) into active caspase 9 (Casp9). It is noteworthy that caspase 3, either activated by GzmB or caspase 9, also reaches the mitochondria – *via* Sam50 – where it cleaves a subunit of ETC complex I, leading to the production of ROS. Following these events, ROS concentration increases in the cytosol of the infected cell. The cytosolic ROS are involved in the translocation of the SET complex from the endoplasmic reticulum (ER) to the nucleus, where it is cleaved by GzmA and turns into a DNA degrading complex. GzmA and GzmB also reach the nucleus where they cleave nuclear substrates, such as lamin B, histones and PARP-1.

intracellular pathogens evolved multiple mechanisms to counteract the death machinery, as already documented in a vast body of comprehensive reviewing literature (87, 94–97). More interesting in this particular context, the Gzms are capable to digest vital microbial substrates independently of host cell death that can directly affect pathogen survival as discussed below in the main focus of this reviewing article.

## NON-CYTOLYTIC ANTIMICROBIAL FUNCTIONS OF THE GRANZYMES

Non-cytolytic, direct antimicrobial activities by the Gzms were primarily demonstrated in virus-infected cells (2). When the Gzms enter a virus-infected cell in a PFN-dependent manner, they will induce apoptosis to deprive the virus of its protective niche as described above. The induction of programed cell death is often inefficient as many viruses evolved multiple pathways to inhibit the death machinery by means of caspase or Gzms inhibition, as well as by mimicking anti-apoptotic proteins, such as Bcl-2 (98–101). Nevertheless, the Gzms can effectively overcome this inhibition by targeting viral proteins or host proteins hijacked by the virus involved in viral replication. The laboratory of Markus Simon previously demonstrated that mouse GzmA cleaved and therefore inactivated the enzymatic activity of reverse transcriptase from Moloney murine leukemia virus. As reverse transcriptase activity is critical for the retroviral life cycle, GzmA might potentially interfere with retroviral replication (102).

In a report concerning adenovirus, GzmH was shown to proteolytically degrade adenovirus DNA-binding protein (DBP), a crucial viral component DNA replication (103). Interestingly, GzmH additionally directly inactivated L4-100K assembly protein crucial for viral assembly and also a potent inhibitor of GzmB (104), suggesting complex interaction of these serine proteases in virus-infected cells.

Also for the family of *Herpesviridae*, such as human cytomegalovirus (HCMV) or herpes simplex virus-1 (HSV-1), multiple viral substrates were identified that are targeted by the Gzms. GzmM interferes with HCMV replication independently of cell death by the proteolytic degradation of phosphoprotein 71, a HCMV protein critical for immediate-early protein expression (105). In more recent works, the same laboratory demonstrated that virus-specific T cells control HCMV replication in a non-cytolytic manner by the Gzms-mediated degradation of the HCMV immediate early proteins IE1 and IE2 (106), as well as of host cell hnRNP K, essential for HCMV replication (107).

GzmA deficiency in mice was associated with impaired control of HSV-1 in infected neurons (108). In addition, human GzmB cleaves the HSV-1 immediate-early protein ICP4, therefore potentially contributing to the non-cytolytic inhibition of viral reactivation in latently infected ganglion cells, mediated by HSV-1 specific T cells (109). In a more recent study, several novel HSV-1 GzmB substrates were identified, suggesting an even broader non-cytolytic role of GzmB in the control of *Herpesviridae* (110).

In conclusion, non-cytolytic, direct antimicrobial activities of the Gzms against viruses are well established. Viral substrates or, for viral replication, essential host factors that are targeted by the Gzms were identified for many additional viruses, such as vaccinia (111), hepatitis C (112) and hepatitis B (113), as well as influenza A virus (114).

Less is known for other intracellular pathogens, such as bacteria or parasites. In earlier work, we found that activating human naïve T cells with bacterial antigens not only triggered the expression of known antibacterial effectors, such as GNLY, interferon-γ or tumor necrosis factor-α, but also of the Gzms in remarkable high levels, in particular GzmB (115). These findings corroborated a previous study indicating elevated plasma levels of GzmA and GzmB in patients with bacterial infections or after endotoxin administration (116). By following up these works, we realized through functional antibacterial assays that GzmA, GzmB and GzmM (the other human Gzms were not tested) are potent antibacterial effectors when these enzymes are delivered into bacteria by GNLY (117, 118) (**Figure 2**). Building on these findings, a recent study demonstrated that the high levels of secreted GzmB and GNLY by activated mucosa-associated invariant T cells (MAIT) not only directly damage bacteria but also increase the susceptibility to carbapenems in multidrug resistant *E. coli* (119, 120). In addition, it was revealed that the three major effector molecules of cytotoxic lymphocytes (Gzms, GNLY and PFN) collaborate in a highly coordinated manner to kill intracellular bacteria, such as *Listeria monocytogenes* (121). The proteolytic activity of the Gzms is necessary to achieve this function, as mutations of their catalytic site impaired the killing of intracellular pathogens. These mutations were introduced by using a mammalian expression system, allowing the generation of comparable purifications of catalytically active and non-active Gzms (122). An unbiased proteomics search for GzmB substrates in several bacterial strains revealed a well-defined list of bacterial proteins, involved in multiple critical metabolic pathways, including protein synthesis and virulence (123). Indeed, extracellular Gzms degraded secreted bacterial virulence factors in absence of GNLY, overall decreasing virulence of the affected bacteria, therefore enabling bystander immune and non-immune cells to more efficiently eliminate the invading pathogens (124).

Interestingly, Gzms-mediated killing mechanisms after delivery by PFN and GNLY were also found against certain unicellular parasites, such as *Plasmodium falciparum* (125, 126). For the *Plasmodium* parasite, we found that the mechanism of Gzms delivery changed upon maturation of the intracellular pathogen in red blood cells (RBCs). While early stage infected RBCs (rings and trophozoites) are susceptible to PFN and resistant to GNLY, late stages (schizonts) display the opposite behavior due to membrane cholesterol depletion and increased phosphatidylserine exposure upon parasite maturation (**Figure 3**) (127). Also for *Toxoplasma gondii* and *Trypanosoma cruzi*, a PFN- and GNLY-dependent delivery mechanism of the Gzms was revealed that induced a death pathway in the parasites,

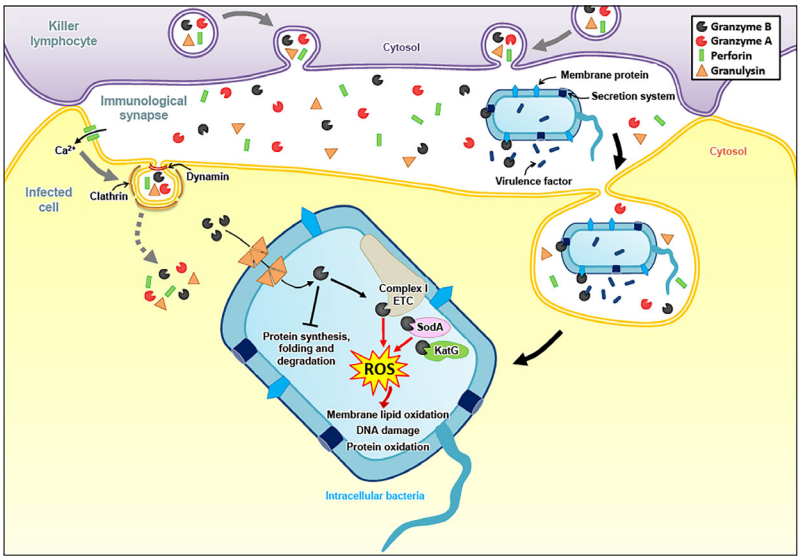


**FIGURE 2** | Granzyme-mediated death pathways in bacteria. To fight intracellular bacteria, Gzms and GNLY are delivered into infected cells in a PFN dependent manner. GNLY then forms pores in bacterial membranes, allowing the entry of the Gzms into the bacterial cytosol. GzmB cleaves the catalytic subunits of electron transport chain (ETC) complex I, as well as bacterial proteins involved in antioxidant defense, generating ROS that induce membrane lipid oxidation, DNA damage and protein oxidation. GzmB also target various bacterial proteins involved in protein synthesis, folding and degradation. Independently of GNLY, extracellular GzmB directly targets external bacterial components, such as secretion systems, membrane proteins and secreted virulence factors to attenuate virulence and, consequently, to facilitate bacterial elimination in bystander cells.

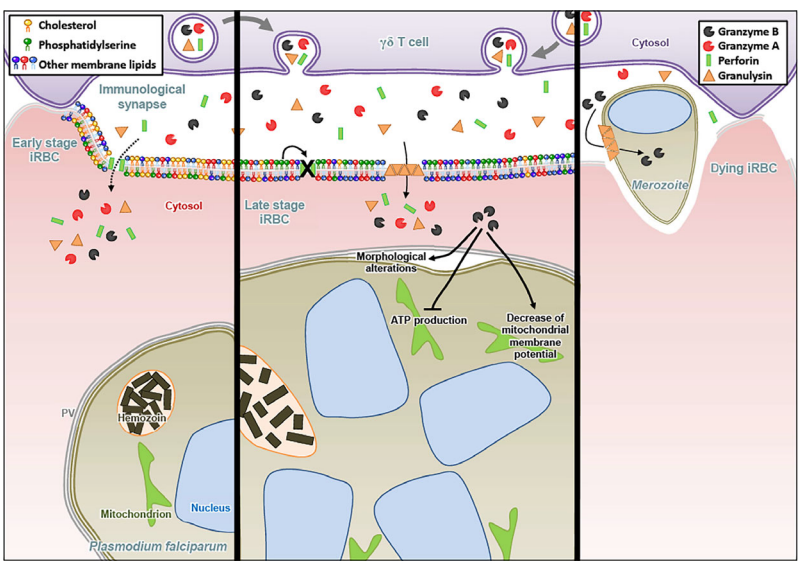


**FIGURE 3** | Granzyme B released by γδ T cells contributes to anti-malaria defense. The particular killer lymphocyte subset bearing the γδ T receptor forms an immunological synapse with *Plasmodium falciparum*-infected red blood cells (iRBC). In early stage iRBC, the plasma membrane contains cholesterol-enriched lipid rafts and the negatively charged phosphatidylserine (PS) is predominantly present in the inner leaflet. At that early stage, PFN can form membrane pores allowing the entry of Gzms, while being resistant to GNLY lysis. For late stage iRBC, cholesterol depletion allows the GNLY to disrupt the membrane while the surface exposure of PS inhibits the formation of PFN pores. Once in the iRBC, GzmB induces dramatic morphological alterations of late stage parasites (schizonts), notably the detachment of the parasitophorous vacuole (PV). Moreover, GzmB inhibits ATP production and decreases the mitochondrial membrane potential of the parasite. At the end of the parasite growth cycle, the rupture of iRBC plasma membrane leads to merozoites egress. GNLY also disrupts the membrane of the merozoite, allowing the entry of GzmB in the parasite.

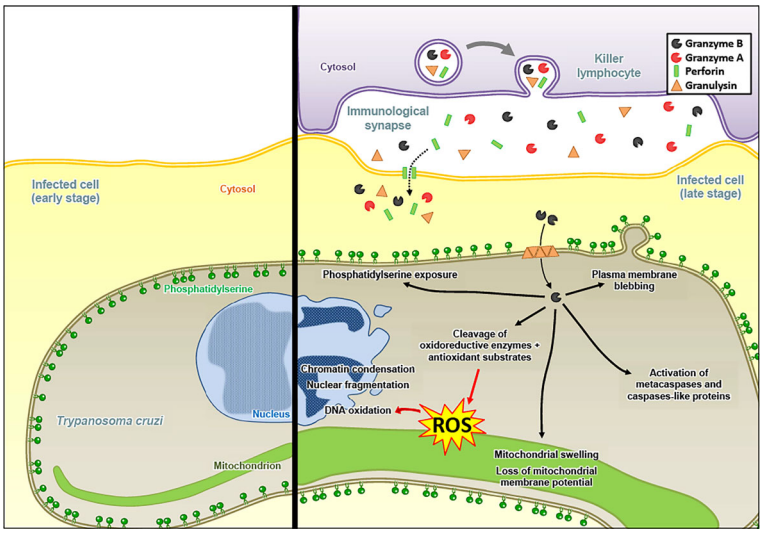


**FIGURE 4** | Granzyme B induces an apoptosis-like death pathway in *Trypanosoma cruzi*. The Gzms of killer lymphocyte granules reach the cytosol of *T. cruzi* in an infected host cell *via* a PFN- and GNLY-dependent delivery mechanism. After entry into the parasites, GzmB targets antioxidant defense substrates, which leads to the accumulation of ROS, responsible for DNA oxidation. GzmB triggers classical mammalian apoptosis features: (1) chromatin condensation and nuclear fragmentation, (2) mitochondrial swelling and loss of membrane potential, (3) plasma membrane blebbing, and (4) phosphatidylserine exposure. GzmB also induces the activation of metacaspases and caspase-like proteins, although it might not be required for GzmB-mediated parasite death.

displaying morphological features highly similar to mammalian apoptosis (**Figure 4**) (128).

## OXIDATIVE ANTIMICROBIAL FUNCTIONS OF THE GRANZYMES

As for the cytolytic activities of the Gzms, the induction of ROS seems to be a critical merging point in the antimicrobial mechanisms against various pathogens. In *E. coli*, the Gzms attacked homologue subunits of the respiratory chain complex-1, as in mammalian mitochondria, suggesting an evolutionary well-conserved killing mechanism. The premature electron leakage from the disrupted respiratory machinery in combination with the GzmB proteolysis of important antioxidant defense enzymes, such as superoxide dismutase and catalase, triggered lethal ROS levels in the affected bacteria (118). GzmB also extensively targeted the ROS defense machinery in the proteome of *Mycobacteria tuberculosis*, *Listeria monocytogenes*, as well as *Salmonella typhimurium*, suggesting that oxidative mechanisms also play a central role in the GzmB-mediated death pathways in these bacterial pathogens (123, 124). Major antioxidant enzymes were also degraded by GzmB in the unicellular parasites, *Plasmodium falciparum, Toxoplasma gondii* and *Trypanosoma cruzi* (125, 128). For the latter parasites, a dominant role of ROS in the killing mechanism was indicated by several lines of evidence: 1. ROS were produced in response to GzmB after delivery with pore forming proteins, 2. ROS scavengers efficiently inhibited the killing, and 3. GzmB uncleavable point mutations in major antioxidant defense enzymes slowed down the death pathway (128). GzmB delivery into unicellular parasites also clearly affect the mitochondria as indicated in morphological alterations, loss of mitochondrial membrane potential and decreased ATP production (125, 128).

## CONCLUSIONS

Though this particular field of research is only developing and further study is necessary, we think it is fair to state that the Gzms exert potent antimicrobial activities by direct proteolysis of vital microbial substrates that are crucial for their replication. Best studied so far in virus-infected cells; however, numerous studies indicate that the Gzms can also lethally affect intracellular bacteria and unicellular parasites by means that are independent of host cell cytolysis. Mitochondria and increased ROS generation seem to be on center stage in Gzms-mediated death pathways in mammalian cells and unicellular parasites. As mitochondria originated from endosymbiotic alpha-proteobacteria, it was not surprising to find respiratory chain disruption by the Gzms in modern living bacteria. To what exact extent these ROS contribute to the killing pathways of the different microbes and mammalian cells is still a matter of debate and needs further study. Nonetheless, there is little doubt that ROS pathways seem to be a highly conserved target in Gzms-mediated death pathways in various species that are evolutionarily far apart.

## AUTHOR CONTRIBUTIONS

MW conceived the concept. ML, MH-C, P-YM, DM and MW researched the literature, wrote, edited and revised the

manuscript. ML designed and illustrated the figures. All authors contributed to the article and approved the submitted version.

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# Siglecs Modulate Activities of Immune Cells Through Positive and Negative Regulation of ROS Generation

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Reactive oxygen species (ROS) are a group of oxygen-containing highly-reactive molecules produced from oxidative metabolic processes or in response to intracellular signals like cytokines and external stimuli like pathogen attack. They regulate a range of physiological processes and are involved in innate immune responses against infectious agents. Deregulation of ROS contributes to a plethora of disease conditions. Sialic acids are carbohydrates, present on cell surfaces or soluble proteins. Sialic acid-binding immunoglobulin-like lectins (Siglecs) recognize and bind to sialic acids. These are widely expressed on various types of immune cells. Siglecs modulate immune activation and can promote or inhibit ROS generation under different contexts. Siglecs promote ROS-dependent cell death in neutrophils and eosinophils while limiting oxidative stress associated with chronic obstructive pulmonary disease (COPD), sickle cell disease (SCD), coronavirus disease-2019 (COVID-19), etc. This review distinguishes itself in summarizing the current understanding of the role of Siglecs in moderating ROS production and their distinct effect on different immune cells; that ultimately determine the cellular response and the disease outcome. This is an important field of investigation having scope for both expansion and medical importance.

**Keywords: chronic obstructive pulmonary disease, coronavirus disease (COVID-19), *Leishmania donovani*, neutrophil, reactive oxygen species, siglecs, Siglec-sialoglycan, eosinophil**

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## INTRODUCTION

Sialic acids (SA), a family of nine-carbon acidic monosaccharides, are commonly found at terminal positions in glycan chains attached to glycoproteins/glycolipids (sialoglycans) present on cell surfaces or soluble proteins. So far, nearly 50 different derivatives of SA have been discovered (1). Sialic acids are commonly found in higher mammals but also in several protozoans, bacteria, and fungi (1). Sialic acid-binding immunoglobulin-like lectins (Siglecs) are a class of glycan-binding proteins that recognize and bind to these SA (2–4). Several Siglecs modulate cellular activity and response *via* signaling through cytoplasmic regulatory motifs.

Reactive oxygen species (ROS) refers to oxygen-derived, highly reactive molecules which include superoxide anion and hydrogen peroxide (5). ROS are produced in mitochondria as by-products of the electron transfer chain during aerobic respiration. Phagocytic cells (macrophages, neutrophils, and

dendritic cells) also generate ROS and reactive nitrogen species (RNS) as components of "oxidative burst" for degrading biomolecules and internalized pathogens (6).

In this review, we have attempted to highlight how Siglec-sialoglycan interactions modulate ROS generation in various immune cells depending on physiological states or infections.

## Distribution and Classification of Siglecs

Most immune cells of hematopoietic origin express one or more kinds of Siglecs (7). Although resting T cells show low Siglec expression, a few T cell subsets express Siglecs (particularly Siglec-5, 7, 9, and 10) after activation or in specific contexts (8–10) (**Table 1**). Some Siglecs, like Siglec-9, are expressed on several immune cells. However, expression of certain Siglecs is restricted to particular cell types; like Siglec-1 (Sialoadhesin) on monocytes, macrophages, and dendritic cells (15). Siglec-2 (CD22) is predominantly observed on B cells though it is also expressed at low levels on mast cells, dendritic cells, and basophils (17–20) (**Table 1**).

Siglecs are classified into two major groups based on sequence homology. The first group (Siglec-1, CD22, Siglec-4, and

**TABLE 1** | Distribution of human and mice siglec families on different cells, ligand preferences and their common functions.

| Siglecs | Distribution on cells | Linkage preference on sialylated ligands | Functions | References |
|---|---|---|---|---|
| **Evolutionary conserved siglecs** | | | | |
| Siglec-1 (CD-169) | Macrophages | α2,3 | Recognition and phagocytosis of sialylated pathogens. Modulates immune response through cell-cell interactions | (7, 11–15) |
| Siglec-2 (CD22) | Predominantly on B-cells, also detected in Dendritic cells Mast cell Basophils, Gut eosinophils of mouse | α2,6- preferably, Neu5Gc/ Neu5Ac | Regulates B cell survival, signaling and homeostasis | (7, 11, 12, 14, 16–20) |
| Siglec-4 (MAG) | Neuronal cells | α2,3> α2,6 | Secures myelin-axon associations through binding with axonic gangliosides GD1a and GT1b. Regulates axon growth and survival | (7, 12) |
| Siglec-15 | Osteoclasts, macrophages | α2,6 | Regulates osteoclast differentiation, involved in regulation of immune response | (11–13, 16) |
| **Siglec-3 (CD-33) related siglecs** | | | | |
| Siglec-3 | Myeloid progenitors | α2,6> α2,3 | Involved in immune inhibitory functions | (7, 11, 12) |
| Siglec-5 | Monocytes, neutrophils, activated T cells | α2,3 | Involved in pathogen phagocytosis and clearance of sialylated substrates | (7, 9, 10, 12) |
| Siglec-6 | Trophoblasts, Mast cells B cells | α2,6- is preferred | Highest expression levels in placenta but function in gestation still not explored | (11, 12) |
| Siglec-7 | Neutrophils, monocytes, mast cells, NK cells, CD8-T cell subset | α2,8- is preferred | Inhibitory in nature, down regulates T cell signaling | (11, 12) |
| Siglec-8 (Human) Siglec-F (closely related functional murine paralog) | Eosinophils, basophils, mast cells Eosinophils Macrophages | α2,3- and sulphated ligands | Involved in cellular apoptosis | (7, 12) |
| Siglec-9 (Human) | Neutrophils, monocytes, NK cells, dendritic cells, B cells, CD8-T cell subset | α2,3 or α2,6- or sulfated ligands | Involved in cellular response apoptosis and inhibition of immune response | (7, 9, 10, 12) |
| Siglec-E (murine paralog) | Neutrophils Monocytes dendritic cells | | | |
| Siglec-10 (Human) | NK cells, B cells, monocytes eosinophils, T cells | α2,3 or α2,6 | Inhibits calcium signalling mediated by B cell receptor | (7, 10, 11) |
| Siglec-G (Murine paralog) | B cells, dendritic cells | | | |
| Siglec-11 | Macrophage, B cells, microglia, ovary stroma | α2,8- is preferred | Involved in pathology of neurodegenerative diseases | (7, 11) |
| Siglec-14 (Human) | Monocytes, neutrophils | α2,3 | Functions as an activating receptor through association with DNAX Activating Protein of 12 kDa (DAP12) | (7, 14) |
| Siglec-H (Murine paralog) | Monocytes neutrophils Microglia | | | |
| Siglec-16 | Microglia | α2,8- is preferred | Involved in Pathology of neurodegenerative disease | (7, 11) |

*This table has been compiled from information collected from references # (7, 9–20). The distribution of siglecs on human and murine immune cells is indicated here.*

Siglec-15) has low sequence similarity between each other but is conserved across species. In contrast, CD33-related Siglecs (CD33r Siglecs) are closely related but not highly conserved (21). Siglecs have been extensively studied in humans, mice, and few other animals. Humans express around 11 different CD33r Siglecs (Siglec-3,-5,-6,-7,-8,-9,-10,−11,−14,−16,−17) while mice express only a few (Siglec-3,-E,-F,-G,-H) (11). Therefore, murine equivalents of only a few human Siglecs are known (**Table 1**).

## Siglec Affinities and Ligand Preferences

Sialic acids are attached to glycans *via* α-glycosidic linkages formed between its C2 with C3 (α2,3) or C6 (α2,6) positions of galactose or C6 position of N-acetylgalactosamine. Polymers of SA are termed polysialic acid, where successive SA residues are primarily linked *via* α2,8 linkages, or by α2,9 linkages in a few cases (1). Different Siglecs have characteristic affinities towards specific sialoglycans based on the SA position, linkages, and surrounding sugars (**Table 1**). Siglec-9 recognizes SA linked to galactose *via* α2,3/α2,6 linkages and other sialylated structures like sialyl Lewis-x (SLex), 6-sulfo-SLex (22, 23). However, Siglec-8 prefers α2,3 linked SA attached to sulfated galactose, as seen in 6-sulfo-SLex (22). Ligand preferences of several Siglecs have been elucidated through glycan-binding arrays, molecular modeling studies, cell-based binding assays, etc. (22). Siglecs can bind sialoglycans present on the same cell (*cis*-interactions) or extracellular ligands present on neighboring cells or secretory glycoproteins (*trans*-interactions).

## Siglec Clustering Enhances Their Signaling Activity

Siglec-sialoglycan binding is weak and transient (24). Interaction between Siglecs with multivalent ligands leads to Siglec clustering, which increases the strength of Siglec-ligand binding and initiates cellular signaling (22, 25–27). Multivalent ligands present several Siglec-binding sites. These may be extracellular ligands, antibodies, synthetic agonists interacting with Siglecs in *trans*, or cell surface sialoglycans, membrane-bound synthetic ligands binding in *cis* (26, 27). Siglec-clustering into nanodomains was revealed by high-resolution microscopy (28, 29). Siglec clustering into signaling domains by anti-Siglec antibodies is shown (**Figure 1A**).

## Function

Siglecs are transmembrane proteins that generally interact with sialoglycans through the carbohydrate recognition domain present in their extracellular *N*-terminal immunoglobulin-like folds (V-set domain). Several Siglecs contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic domains while some are associated with adaptor proteins that contain immunoreceptor tyrosine-based activatory motifs (ITAMs) (7, 30). Signaling *via* ITIM leads to recruitment of Src homology region domain-containing phosphatase-1 and 2 (SHP-1, SHP-2) which inhibits phosphorylation-based cellular signaling pathways (14, 31). Siglec-sialoglycan binding leads to modulation of cellular activity due to signaling *via* either ITIMs (inhibitory) or ITAMs (activatory) motifs (21).

Siglecs play several roles in normal physiology, including immunomodulation, phagocytosis of sialylated pathogens (mainly Siglec-1); regulation of B cell signaling and survival (CD22); maintenance of axon-myelin interactions, axon growth (Siglec-4); regulation of osteoclast differentiation (Siglec-15), etc (12–14, 16). Distribution, ligand preference, and functions of a few human Siglecs have been compiled in **Table 1**.

## Effect of ROS

ROS has a direct detrimental effect on lipids, proteins, and DNA. However, low levels of ROS are required for maintaining metabolism, signal transduction, cell proliferation, apoptosis, and aging process (32). Oxidative stress due to excessive ROS generation is implicated in asthma, chronic obstructive pulmonary disease (COPD), diabetes, cardiovascular diseases, auto-immunity, and neurodegenerative diseases (5). Phagocytic cells express a multi-subunit NADPH-dependent phagocytic oxidase (Phox or NOX2), which produces ROS as a component of antimicrobial defense (33). Some components of NOX2 ($p67^{phox}$, $p47^{phox}$, $p40^{phox}$, a small G protein-rac1, rac2), are localized in the cytosol and some are membrane-associated ($gp91^{phox}$, $p22^{phox}$). The activity of membrane-associated $gp91^{phox}$ (34) is indispensable for NOX2 function, which uses NADPH as an electron donor to generate superoxide anion ($O_2^{\bullet-}$) by oxygen reduction (35–37).

## Siglec-Based Modulation of ROS Production

Siglecs modulate ROS production through Siglec-sialoglycan binding, signaling through ITIMs or direct protein-protein interactions. Siglec-9 was identified as a potential ligand for human amine oxidase type 3 (hAOC3) by phage peptide library-based screening (38). Flow cytometry confirmed the binding between recombinant Chinese Hamster Ovary cells (CHO) expressing hAOC3 and CHO-expressing Siglec-9 (39). Surface plasmon resonance (SPR) based binding assays demonstrated that Siglec-9 $C2_2$ domain binds to the active site of hAOC3. Introduction of point mutations followed by SPR-based binding assays identified Arg284 and Arg290 in Siglec-9 to be critical for its binding with hAOC3. Such Siglec-binding with oxidase enzyme through protein-protein interactions enhances hAOC3 activity and generates hydrogen peroxide (39). Siglec-9 also binds to sialoglycoprotein hAOC3 through sialic acid-Siglec binding *via* its V domain (40).

Siglec-E knockout neutrophils infected with non-sialylated *Escherichia coli* strains (Gram-negative bacteria) produced significantly less amount of ROS than wild-type neutrophils, suggesting that Siglec-E promoted ROS generation (41). Silencing Siglec-E or Siglec-9 (the human equivalent of Siglec-E) also reduced ROS generation in neutrophils/THP-1 cells after *E.coli* infection (41). Immunoprecipitation revealed that endogenous Siglec-E associates with NOX2 subunits ($gp91^{phox}$/$p47^{phox}$) in *E.coli*-infected murine neutrophil and Tyr432 residue was found to be critical for Siglec-E-$p47^{phox}$ binding. Cells overexpressing Siglec-E with mutated Tyr432 also produced lower amounts of ROS following *E.coli* infection.

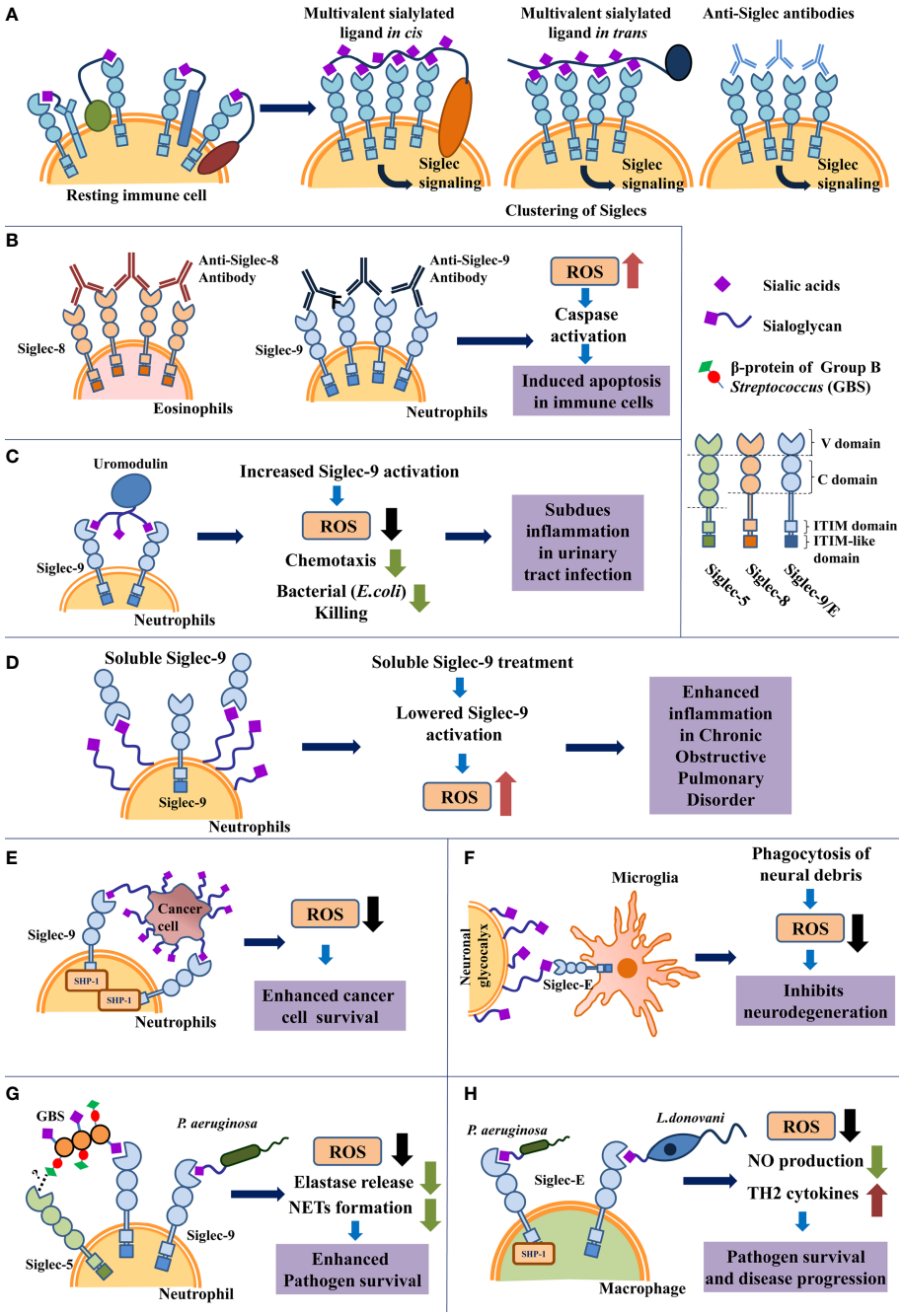


**FIGURE 1** | Schematic representation of ROS modulation by Siglecs in immune cells under different pathological conditions. **(A)** Schematic representation of clustering of Siglecs on immune cell surface induced by interaction with various multivalent ligands. **(B)**. Siglec-crosslinking using anti-Siglec antibodies promote ROS generation which triggers cellular apoptosis in resting neutrophils and eosinophils. **(C)** Uromodulin binding leads to enhanced Siglec-9 activation in neutrophils *in vitro*, inhibiting ROS production. This interaction possibly limits excessive inflammation during urinary tract infection. **(D)** Increased expression of soluble Siglec-9 (sSiglec-9) lowers Siglec-9-sialic acids engagement on neutrophils, leading to increased inflammation and ROS generation in chronic obstructive pulmonary disease (COPD). **(E)** Cancer cell sialoglycoproteins engage Siglecs on neutrophil *via* Siglec-sialoglycan interaction to reduce ROS production, which enhances cancer cell survival. **(F)** Siglec-E engagement with neural sialoglycoproteins suppresses ROS generation by microglial cells and prevents oxidative stress mediated neuro-degeneration. **(G)** Sialylated bacteria like Group B *Streptococcus* (GBS) and *Pseudomonas aeruginosa* interact with Siglec-9 present on neutrophil using sialic acids present on their surface. Additionally, non-sialylated GBS β-protein of GBS also binds with Siglec-5 present on neutrophil in a sialic acid-independent manner. Both Siglec-sialoglycan and Siglec-protein interactions encourage pathogen survival through subdued immune response. However, the exact binding site of protein mediated interaction between Siglec-5 and GBS β-protein, remains unknown. **(H)** Interaction of macrophage Siglec-E with sialylated pathogens like *Pseudomonas aeruginosa* and *Leishmania donovani* respectively subdues ROS production for disease progression.

This confirmed that Tyr432 in the ITIM domain of Siglec-E is needed for its association with NOX2 subunit $p47^{phox}$ which promotes ROS production (41).

Interestingly, treatment of primary human neutrophils with a synthetic Siglec-9 agonist (pS9L) or anti-Siglec-9 antibody also leads to ROS generation which is inhibited upon SHP-1/2 inhibitor treatment. This indicates that Siglec-9 engagement also promotes ROS generation through SHP-1/SHP-2 signaling (42).

## Siglec Engagement on Immune Cells Modulates ROS Generation

### Eosinophils

Treating resting eosinophils with anti-Siglec-8 monoclonal antibodies and secondary polyclonal antibodies leads to extensive crosslinking or clustering of Siglec-8 (**Figure 1B**, left panel). Siglec-8 clustering was followed by ROS generation, reduction in mitochondrial membrane potential, and cleavage of caspases; culminating in cellular apoptosis (43, 44). Eosinophils incubated with pro-survival cytokine IL-5 show further increased cell death upon Siglec-8 cross-linking (45). These IL-5-activated eosinophils exhibit caspase-independent necrotic death, involving ROS generation and increased phosphorylation of MEK1, ERK1/2 (46). Treatment with ROS inhibitors confirmed that Siglec-crosslinking-mediated ROS production is essential for triggering eosinophil death in both resting and activated cells. Produced ROS was accumulated intracellularly in eosinophils (47).

In contrast, eosinophils stimulated with pro-survival cytokine IL-33 also exhibit enhanced cell death after Siglec-8 cross-linking, but without any significant increase in ROS generation (48). The lack of ROS production in IL-33-stimulated eosinophils remains to be explored.

Siglec-F shows a similar ligand binding profile like Siglec-8 and is also expressed on eosinophils. It is considered as a functionally convergent paralog of Siglec-8 in mice (49, 50). Mice treated with anti-Siglec-F antibodies show induction of caspase-dependent eosinophil death independent of NADPH oxidase activity and ROS production (51).

Binding Siglec-8 with monoclonal antibodies or synthetic Siglec-8 ligands leads to increased expression of CD11b/CD18. CD11b/CD18 heterodimer belongs to the β2-integrin subgroup, which increases the adhesiveness of IL-5-stimulated eosinophils. Such Siglec-8 engagement also leads to a time-dependent increase in ROS production, dependent on β2-integrin expression (52). NADPH oxidase (NOX) enzyme was identified as the source of ROS (52). This indicates antibodies or ligand-based Siglec-8 binding triggers ROS production through probable modulation of NOX activity.

### Neutrophils

Neutrophils are the most abundant type of immune cells present in blood. Neutrophils perform immunosurveillance and respond to infiltrating microbes by phagocytosis, respiratory burst, degranulation, and formation of neutrophil extracellular traps (NETs) (53). Following successful clearance of microbes, responding neutrophils generally undergo apoptosis. However, pro-survival cytokines or growth factors in the milieu often prevent such apoptosis, leading to chronic or acute inflammations.

Antibody-based cross-linking of Siglec-9 triggers apoptosis in normal resting neutrophils due to ROS generation and caspase cleavage (**Figure 1B**, right panel) (54). Interestingly, neutrophils isolated from patients suffering from inflammatory conditions like rheumatic arthritis, acute septic shock, etc. show enhanced expression of Siglec-9 and increased death upon Siglec-9 cross-linking. Neutrophils stimulated with GM-CSF, interferon-α or interferon-γ (IFN-α/γ) similarly exhibit increased cell death after Siglec-9 cross-linking (47).

Such cytokine-stimulated neutrophils mainly showed non-apoptotic cell death upon Siglec-binding, characterized by cytoplasmic vacuolization, non-involvement of caspases, and only ROS generation. However, ROS generated by NADPH oxidase was identified to be crucial in both kinds of cell death (54).

Neutrophils express adhesion factors (like CD11b containing β2-integrins) for attachment and migration from blood to other sites of injury or infection. They adhere to fibrinogen-coated plates *via* CD11b-fibrinogen binding, leading to integrin-triggered ROS production (55). Additionally, Siglec-E binding with the sialoglycoprotein fibrinogen further increases ROS generation *via* NADPH-oxidase activity, which required fibrinogen-CD11b binding. In summary, Siglec-8 in eosinophils (52) and Siglec-9/E in neutrophils (54, 55) modulate NOX activity for promoting ROS generation.

Additionally, Siglec-E-mediated β2-integrin-dependent ROS generation prevented migration of neutrophils into the lungs of mice exposed to lipopolysaccharide, which was reversed upon inhibition of NOX activity (55).

### In Inflammation

In normal physiology, Siglec-sialoglycan mediated signaling modulates immune cell activation, their response, ROS release, etc for preventing excessive inflammation and tissue injury (6).

### Urinary Tract Infections (UTI)

Tamm-Horsfall protein (THP, uromodulin), is the most abundant protein in urine. It is a sialoglycoprotein that binds to Siglec-9/Siglec-E. Uromodulin treatment of neutrophils leads to reduced ROS generation, lowered chemotaxis, and reduced killing of uropathogenic *Escherichia coli* (56). During urinary tract infections, Uromodulin-Siglec-9 interaction possibly limits excessive neutrophil infiltration, reduces ROS generation, thereby modulating tissue inflammation (**Figure 1C**).

### Chronic Obstructive Pulmonary Disorder (COPD)

Siglec-based regulation of ROS levels is observed in COPD patients. These patients exhibit elevated levels of the extracellular domain of Siglec-9 (soluble Siglec-9/sSiglec-9) in their plasma along with neutrophil hyperactivation, chemotaxis, and increased oxidative stress (57) (**Figure 1D**). Healthy neutrophils treated with sSiglec-9 *in vitro* show lower binding between neutrophil surface Siglec-9 and sialoglycans and thereby reducing Siglec-9 activation. This leads to

increased ROS production and chemotaxis by neutrophils (57). Expression of Siglec-9 was upregulated in peripheral blood and alveolar neutrophils in COPD patients, possibly to enhance Siglec-based inhibitory signaling in response to neutrophil hyperactivation (57).

### Sickle Cell Disease (SCD)

Siglec-based regulation of neutrophil activation is also seen in sickle cell disease (SCD). Neutrophils cultured with healthy erythrocytes showed lowered activation and ROS generation. Healthy erythrocytes suppress neutrophil activation by engaging with neutrophil Siglec-9 *via* sialylated erythrocyte membrane proteins like glycophorin A (58). In SCD, erythrocytes rapidly age, losing membrane elasticity and undergoing changes in cell membrane composition and protein expression. SCD-erythrocytes contain more SA compared to healthy erythrocytes but show lowered binding with neutrophil-Siglec-9 (59). Consequently, culturing healthy neutrophils with SCD erythrocytes leads to increased ROS release due to lowered Siglec-9 activation. Hyperactivation of neutrophils may lead to systemic inflammation, vaso-occlusion, etc. commonly observed in SCD.

### Cancer Progression

Several types of cancers exhibit increased sialylation (60, 61). Neutrophils incubated with cancerous cells *in vitro* showed enhanced Siglec-9 engagement with cancer cell sialoglycoproteins, which increased SHP-1 recruitment. Consequently, ROS production and killing of tumor cell was inhibited (62) (**Figure 1E**). Mice lacking Siglec-E (murine equivalent of Siglec-9) show increased neutrophil activity, immunosurveillance, and killing of injected tumor cells (62).

Surprisingly, established tumors grew quicker in mice lacking Siglec-E. Tumor-infiltrating macrophages in such mice showed an M2 type phenotype. Depending on environmental stimuli, macrophages are activated into M1 (pro-inflammatory, eliminates tumor cells) or M2 (promotes cell proliferation, wound repair) macrophages (63). Peritoneal macrophages from mice lacking Siglec-E showed upregulation of M2 polarization markers upon co-culture with cancerous cells. Therefore, depending on the stage of cancer progression, the absence of Siglec-E can enhance cancer cell killing (by neutrophil activity like ROS generation) or promote tumor growth (by inducing macrophage M2 polarization).

### Coronavirus Infections

During the COVID-19 pandemic, coronavirus infections have been linked with uncontrolled inflammatory responses, hyperactivation of neutrophils, and NETs formation (64). Incubation with serum/plasma from COVID-19 patients triggered NETosis in neutrophils from healthy donors (65, 66). Siglec-9 engagement inhibits neutrophil activity and induces apoptosis. A Siglec-9 agonist, a glycopolypeptide bearing modified SA residues and lipid moieties (pS9L), induces Siglec-9 clustering through *cis* interactions in macrophages after membrane insertion (28). Incubation of neutrophils with this agonist leads to ROS generation *via* SHP-1/2 and blocked NETosis induced by COVID-19 plasma or TLR 7/9 agonists (42). This agonist may reduce inflammation by blocking NETosis and by triggering neutrophil apoptosis through ROS generation (54).

### Polysialic Acid-Based Nanoparticles

Polysialic acids serve as multivalent ligands and activate Siglecs. Treatment of neutrophils with phorbol 12-myristate 13-acetate (PMA) induces NETosis and ROS generation. However, incubating neutrophils with aliphatic amine latex nanoparticles coupled to polysialic acids having <9 sialic acid residues, leads to a reduction in PMA-induced ROS levels and NETs formation (67). These polysialylated nanoparticles possibly function as ligands for neutrophil surface Siglec-5. Similarly, injection of polysialic acids having ~20 SA residues in transgenic mice prevented ROS generation by Siglec-11-expressing phagocytes. Age-related macular degeneration happens due to excessive ROS production, complement deposition which may be prevented by targeting siglec-11 *via* polysialic acids (68).

All these studies suggest that enhancing Siglec-sialoglycan engagement may be beneficial for controlling ROS levels in inflammatory disorders.

### Neurodegenerative Diseases

Microglia are immune cells resident in the central nervous system, responsible for detecting invasive pathogens and maintaining tissue homeostasis by removing damaged, apoptotic or unnecessary synapses, neurons, and plaques. Microglial cells produce ROS following the phagocytosis of apoptotic bodies. However, oxidative stress in the central nervous system leads to neurodegenerative problems. SA content is highest in mammalian brains. Silencing Siglec-E expression in microglia confirmed that microglial Siglec-E binds to the sialylated neuronal glycocalyx to suppress ROS release following phagocytosis of neuronal debris (69). Here, Siglec-E regulates microglial ROS to prevent neurodegeneration (**Figure 1F**).

### Early Aging

The lifespan of mammals is positively correlated with the number of CD33r Siglec genes (70). Siglecs are responsible for regulating ROS, particularly produced by activated phagocytes *via* NOX enzyme, during inflammatory responses. ROS possibly plays a role in early aging (71). Deletion of Siglec-E in mice resulted in shortened lifespan with increased generation of ROS and inhibition of ROS-detoxification systems (70). This correlation was confirmed across 26 species and held true for both activatory and inhibitory Siglecs (72).

Although a positive correlation exists between mammalian lifespan and siglecs, further research is needed to clearly understand how both the activatory and inhibitory siglecs are able to regulate the specific signaling pathways and receptors involved in neurodegenerative diseases.

### Down-Regulation of ROS in the Infection Process Through Siglec-Sialoglycan Interaction

The capsular cell wall on Group B *Streptococcus* (GBS) is sialylated and associated with its virulence (73–77). These

sialoglycans interact with Siglec-9 on human neutrophils for suppressing neutrophil oxidative burst and NETs formation, thus leading to enhanced bacterial survival (**Figure 1G**). A similar interaction was also reported with Siglec-E expressed on murine macrophages (76, 78).

Additionally, Group A *Streptococcus* expresses high molecular weight hyaluronan (HMW-HA). A similar sialylated molecule is also expressed on human neutrophils. Thus, Group A *Streptococcus* activates Siglec-9 on neutrophils through molecular mimicry to subdue ROS production, decrease NETs and apoptosis, for securing its existence inside the host (76, 79).

A few GBS strains express a non-sialylated β-protein docked on their cell wall that can efficiently engage Siglec-5 (**Figure 1G**). Such interaction was confirmed through the binding of SA-deficient mutant GBS with hSiglec-5-Fc. Trypsin treatment prevents this interaction by degrading the β-protein on the pathogen, indicating that binding was protein-mediated and independent of SA (75). It remains to be explored how non-sialylated proteins bind to Siglecs, as the binding site and the mechanism of such binding is still unknown. This association activated the inhibitory signaling through the engagement of SHP1/2. Thus, suppressed host immune responses like oxidative burst, repressed phagocytic activity, and extracellular traps in the leucocytes, thereby favoring pathogen persistence (75).

Likewise, we had reported the presence of SA in *Pseudomonas aeruginosa* (PA) and identified a few sialoglycoproteins by mass spectrometry (80). These sialoglycoproteins interact with several human Siglecs *via* SA to enhance their pathogenicity (81–83). Interaction of *PA*-SA with inhibitory Siglec-9 on neutrophils reduced ROS production, elastase release, and decreased NETs formation, which altogether suppressed the activation of these immune cells (**Figure 1G**). Moreover, the association of the bacterial SA with murine Siglec-E on macrophages exhibited enhanced phagocytosis but reduced oxidative burst (**Figure 1H**) (83).

Our group also had demonstrated that *Leishmania donovani*, the causative agent of Indian visceral leishmaniasis displays different derivatives of sialic acid on its cell surface (84). Subsequently, it was also shown that various strains of *Leishmania* species causing different forms of the disease exhibited a differential distribution of SA on their surface. Binding studies of sialylated virulent *L.donovani* strain with soluble siglec-Fc chimeras displayed its high interaction only with Siglec-1 and Siglec-5. Furthermore, we also reported that these parasites interact with Siglec-E on murine macrophages to subvert the host immune response (85, 86). ROS production and other macrophage effector functions were upregulated by silencing Siglec-E, which ultimately diminished the parasite survival inside the host (83). This indicates that Siglec-E suppresses ROS production in parasite-infected macrophages (**Figure 1H**).

All this information supports the well-established immune-inhibitory role of Siglecs in the promotion of pathogen survival and disease progression. Thus, Siglecs at the host-pathogen interface can play a very important role in modulating the immune response through regulating ROS production in the immune cells.

## DISCUSSION

The crucial role of Siglec-sialoglycan interactions in regulating immune and inflammatory responses is increasingly becoming relevant. Siglecs are being recognized as potential therapeutic targets in various inflammatory disorders and cancer (87, 88). Strategies to target activatory or inhibitory Siglecs for regulating immune response involve modulation of overall sialylation, use of antibodies, or sialic acid mimetics (89–93).

Several studies demonstrate that Siglec-sialoglycan interactions additionally promote as well as inhibit ROS generation to control diverse cellular functions ranging from apoptosis to maintenance of cellular life-spans. Such interactions may be beneficial for modulating oxidative stress as a part of anti-tumor, anti-inflammatory therapy. Siglecs may induce immune-tolerance or suppress inflammation by depleting ROS-producing cells or inhibiting ROS release. A few Siglec agonists which enhanced Siglec-based suppression of ROS production in inflammatory disorders have already been demonstrated (28, 42, 67–69).

However, the consequence of Siglec-sialoglycan interaction may vary depending upon the location of Siglec and the presence of its cognate ligand in the surroundings. Also, some of the Siglecs have redundant roles. More comprehensive studies are required to define the outcome of such signaling events before siglecs may be used as therapeutic agents. In this respect, the role of Siglec-sialoglycan interactions and protein-protein interactions in Siglec-based ROS regulation needs to be explored in detail.

## AUTHOR CONTRIBUTIONS

All the authors enlisted have made substantial, direct, and intellectual contribution to the work and approved it in its final form.

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REVIEW
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# Reactive Oxygen Species: Do They Play a Role in Adaptive Immunity?

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The immune system protects the host from a plethora of microorganisms and toxins through its unique ability to distinguish self from non-self. To perform this delicate but essential task, the immune system relies on two lines of defense. The innate immune system, which is by nature fast acting, represents the first line of defense. It involves anatomical barriers, physiological factors as well as a subset of haematopoietically-derived cells generically call leukocytes. Activation of the innate immune response leads to a state of inflammation that serves to both warn about and combat the ongoing infection and delivers the antigenic information of the invading pathogens to initiate the slower but highly potent and specific second line of defense, the adaptive immune system. The adaptive immune response calls on T lymphocytes as well as the B lymphocytes essential for the elimination of pathogens and the establishment of the immunological memory. Reactive oxygen species (ROS) have been implicated in many aspects of the immune responses to pathogens, mostly in innate immune functions, such as the respiratory burst and inflammasome activation. Here in this mini review, we focus on the role of ROS in adaptive immunity. We examine how ROS contribute to T-cell biology and discuss whether this activity can be extrapolated to B cells.

**Keywords: reactive oxygen species, adaptive immunity, T lymphocytes, B lymphocytes, tumor microenvironment**

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## INTRODUCTION

Reactive oxygen species (ROS) include both radical and non-radical species and are formed by the partial reduction of oxygen. The radical species, e.g., superoxide anion ($O^{2-\cdot}$), hydroxyl radical (.OH), and nitric oxide (NO), have unpaired electrons (1, 2). In contrast, the non-radical products, e.g., hydrogen peroxide ($H_2O_2$), hypochlorous acid (HOCl), and peroxynitrite ($ONOO^-$), do not have unpaired electrons but remain powerful oxidizing agents (1). Interestingly, cellular enzymatic systems such as the nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidases, the myeloperoxidases, the nitric oxide synthases (NOS), the monooxygenase activity of cytochrome P450, xanthine oxidase, monoamine oxidase (MAO) and the mitochondrial respiratory chain are sources of the primary radical species ($O^{2-\cdot}$, NO, and $H_2O_2$) (1, 3). At low concentrations, which can be handled by the cellular antioxidant system, $O^{2-\cdot}$, NO and $H_2O_2$ are necessary for signal transduction, cell migration, cell differentiation, cell proliferation, vasoconstriction, inflammation, senescence and aging (4–14). This can be explained, in part, by the fact that to some extent primary species reactions with biomolecules are reversible and they are easily controlled by enzymatic and non-enzymatic antioxidant molecules of the cell antioxidant machinery (15–18).

Interestingly, although even at high concentrations $O^{2-}$, NO and $H_2O_2$ are not directly damaging to cells, they react with themselves or with metal ions to produce the extremely toxic secondary reactive species.OH, $ONOO^-$ and HOCl. These secondary species are poorly controlled and rapidly and irreversibly react with virtually all classes of biomolecules causing oxidative damage. The accumulation of ROS can lead to a state of oxidative stress when the endogenous antioxidant machinery of the cell is overwhelmed (19–24). Consequently, the cells accumulate oxidative damage within the DNA, lipids and proteins, causing cellular dysfunction and cell death (19–23). Excessive ROS production plays a major role in the initiation and amplification of cell death by modulating many signaling pathways. Consequently, ROS levels are contributing determinants for various forms of cell death, including apoptosis, necrosis/necroptosis, ferroptosis, pyroptosis and autophagic cell death (25–32).

The immune system has the unique ability to distinguish self from non-self to protect the host organisms from a plethora of microorganisms and toxins (33–35). It eliminates foreign entities (pathogens and toxins) but tolerates the self (host's own tissues) and its associated microbiota (33, 36, 37). The innate immune system, the components of which are already present before any pathogenic intrusion, is fast acting. It relies on anatomical barriers (the skin and the mucosa lining the respiratory, gastrointestinal and urogenital tracts) to prevent foreign entities from entering the organism (33, 34). These anatomical barriers are reinforced by soluble factors (complement system, pentraxins, collectins and the defensins antimicrobial peptides) as well as by leukocytes (macrophages, dendritic cells, mast cells, neutrophils, eosinophils, natural killer [NK] cells) that neutralize pathogens or kill the infected cells (33, 34). The innate immune system is activated by the recognition of antigenic determinants common to a wide spectrum of microbes (the pathogen associated molecular patterns [PAMP]) and leads to a state of inflammation to alert and combat the ongoing infection (33, 34, 38). Importantly, the activated innate immune system delivers the antigenic information of the invading pathogens to activate the slower but highly potent and specific second line of defense known as the adaptive immune system. The adaptive immune response calls on T lymphocytes and B lymphocytes as, respectively, the effectors of the cellular adaptive immune response and as the antibody-producing cells with the essential functions of eliminating pathogens and establishing immunological memory (35, 39, 40).

ROS have been implicated in many aspects of the immune response to pathogens mainly related to innate immunity. Indeed, they have been proposed to be the common determinant of inflammasome activation, which is critical in the inflammatory process and thus necessary for an efficient immune response. ROS are also essential for pathogen killing by phagocytic cells, as illustrated in chronic granulomatous disease (CGD), an inherited disorder of NADPH oxidase characterized by recurrent and severe bacterial and fungal infections as phagocytes from these patients cannot do the respiratory burst. Here in this mini review, we focus on the role of ROS in adaptive immunity. We examine how ROS contribute to T-cell biology and briefly discuss whether these activities can be extrapolated to B cells.

## ROS AND LYMPHOCYTE ACTIVATION

The engagement of the B-cell receptor (BCR) or T-cell receptor (TCR) provides the specific signal 1, which in association with signal 2 coming from the co-costimulatory receptors, triggers intracellular phosphorylation cascades (**Figure 1**). This results in activation of the transcription factors activator protein 1 (AP1), nuclear factor (NF)-κB, nuclear factor of activated T cells (NFAT), Oct binding factor (OBF)-1/OCA-B (OCA-B/OBF-1 and Pip/interferon regulatory factor (IRF)-4, which are critical for T and B lymphocyte activation (**Figure 1**) (35, 41–45). Early research demonstrated that ROS scavengers such as N-acetyl cysteine (NAC) inhibit NF-κB activation following exposure to phorbol 12-myristate 13-acetate, tumor necrosis factor (TNF)-α, or interleukin-1 (IL-1), indicating that ROS are involved in physiological activation pathways (46, 47).

## ROS CONTRIBUTE TO TCR SIGNALING

Actually, within minutes of TCR stimulation, there is a production of both $O^{2-}$ and $H_2O_2$, which seems to originate from different TCR signaling pathways (48). Specifically, studies in Jurkat T cells showed that ROS increase the phosphorylation and activity of p56lck, ZAP-70, protein kinase C (PKC) and intracellular $Ca^{2+}$ levels (**Figure 1**) (49–51). This results in a phosphoinositide-3 kinase (PI3K)/AKT/mTOR-, Myc- and ERRα-dependent augmentation of the global metabolism (52–56). It was further demonstrated that T cells from $p47^{phox}$-deficient mice do not undergo TCR-induced $H_2O_2$ production, whereas TCR-induced $O^{2-}$ is unaffected following TCR stimulation in these cells, indicating that $H_2O_2$ originates from a lymphocyte-encoded NADPH oxidase (NOX) (57). Additionally, using superoxide scavenger treatments or superoxide deficiency in OT-II.$Ncf1^{m1J}$ mice having CD4 T cell-specific superoxide deficiency, it was shown that superoxide is necessary for Th1 responses as well as IL-12R and proinflammatory chemokine ligand expression in CD4 T cells (58, 59). In fact, in T cells, ROS contribute not only to proximal but also to distal signaling pathways and modulate the activities of transcription factors NFAT, AP-1, and NF-kB to induce gene expression (60, 61). Activated T cells take up large amounts of glucose and produce lactate, indicating that they are primarily glycolytic (61, 62). Interestingly, during CD4 T-cell stimulation, mitochondrial oxygen consumption increases as an indication that mitochondrial function is also important for T-cell activation not only to support the glutamine requirement of these cells but also as a source of ROS (**Figure 1**) (61, 63). It was even shown that mitochondrial ROS specifically from respiratory chain complex III are required for CD4+ and CD8+ T cell expansion *in vivo* (61). Deletion of the Rieske iron sulfur protein (RISP), a subunit of mitochondrial complex III in T cells, resulted in a lack of oxidative phosphorylation and complex III-dependent ROS production and no expression of IL-2 upon CD3/CD28 stimulation (61). This phenotype was rescued by the addition of exogenous $H_2O_2$, clearly demonstrating the ROS requirement for full activation of the CD4 T cells (61). In this context, mitochondrial ROS were downstream of the TCR-mediated cytosolic and mitochondrial

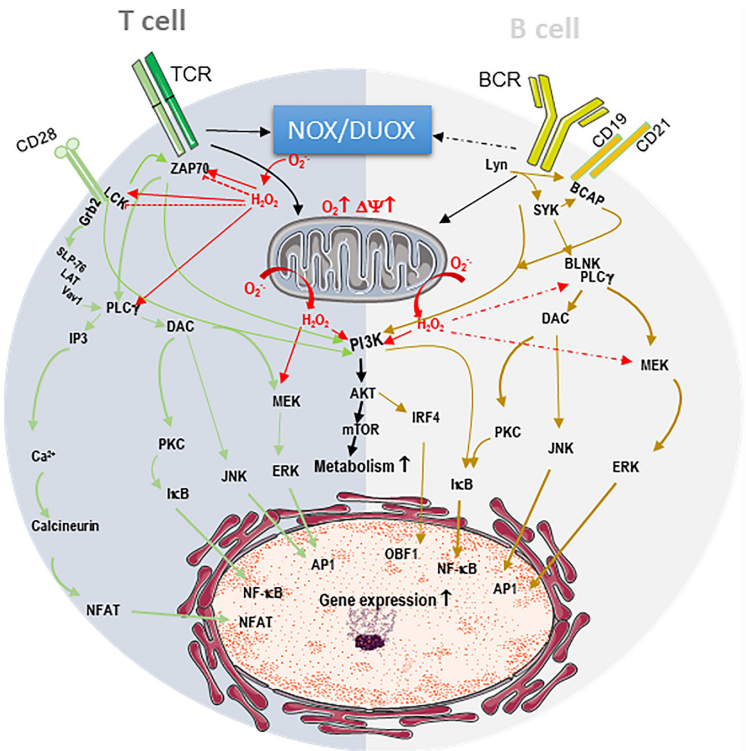


**FIGURE 1** | Endogenous ROS contribute to T and B cell receptor signaling. The engagement of the B-cell receptor (BCR) or T-cell receptor (TCR) and their respective co-receptors CD28 and CD19/CD21 triggers intracellular phosphorylation signaling cascades resulting in the activation of transcription factors AP1, NF-κB, NFAT, OCA-B/OBF-1 IRF-4, which are critical for T and B lymphocyte activation. In T cell, dark grey, TCR/CD28 stimulation induces the phosphorylation and activation of the kinases p56lck (LCK) and ZAP-70, the phospholipase Cg (PLCg). CD28 recruits growth factor receptor bound protein 2 (Grb2), which docks the complex formed by SH2 domain containing leukocyte protein of 76kDa (SLP-76)/Linker for activation of T-cells (LAT)/signal transducer protein Vav1. The latter complex recruitment closer to the membrane facilitating its activation by ZAP70. PLCg generates inositol 3 phosphate (IP3) to mobilize intracellular $Ca2^{+}$ stores resultingin the activation of the phosphatase calcineurin. PLCg also generates diacylglycerol (DAC) to activate protein kinase C (PKC), c-Jun N-terminal Kinase (JNK) and mitogen-activated protein kinase/extracellular signal-regulated kinases (MEK/ERK) cascade. Calcineurin dephosphorylates nuclear factor of activated T cells (NFAT), allowing its nuclear translocation. PKC allows NF-κB nuclear translocation by removing of the inhibitor (IkB), while JNK and ERK activate AP1. In B cells, light grey, BCR/CD19/CD21 stimulation initiates a phosphorylation cascade starting with the activation of the kinases Lyn and SYK, leading to the activation of B cell linker protein (BLNK), B Cell adaptor molecule for phosphoinositide 3-Kinase (PI3K) (BCAP) and PLCg, ultimately resulting in PKC, JNK and ERK activation. Both TCR and BCR signaling potentiate mitochondrial respiration and activate metabolic pathways through their action on the complex formed by PI3K, protein kinase B (PKB/AKT) and mammalian target of rapamycin (mTOR). These signaling cascades are potentiated by ROS (H2O2 and O2-.) from NADPH oxidases (NOX/DUAOX) and from the mitochondria (red arrows). In some instances, NOX triggers the oxidative modification of ZAP70 and LCK to precipitate their degradation and blunt activation (dashed bloc red line).

calcium increase, in agreement with the calcium dependency of mitochondrial TCA cycle dehydrogenases that fuel the electron transport chain (ETC) to increase mitochondrial membrane potential and ROS production (64, 65). Interestingly, mitochondrial ROS are also necessary for CD8 T-cell activation, as inhibition of the respiratory chain complex I decreases the production of $H_2O_2$, calcium flux, and ERK1/2 phosphorylation and impairs CD8 T-cell activation and proliferation (66). Complex I inhibition not only decreases activation of naive cells but also decreases interferon (IFN)-γ and TNF-α production as well as degranulation of effector and memory CD8+ T cells isolated from lymphocytic choriomeningitis virus-infected mice (66).

It is worth noting that some studies suggest mitochondria more than NADPH oxidase are the essential source of ROS involved in the activation process (61–66). This apparent discrepancy could come from the activation status (naïve/primed) or developmental state (CD8/CD4, T helper 1/2/9/17 or regulatory CD4 T cells) considered, which might have different ROS requirements for full activation (67). Nevertheless, collectively these results showed that ROS play a role in the activation and maturation of both CD8 and CD4 T cells (57, 61, 66, 68, 69). Mechanistically, by inhibiting phosphatases, ROS might tilt the balance toward phosphorylation, ultimately potentiating the activation of kinase cascades and transcription factors such as NFAT, which is critical for IL-2 production (61).

Interesting, although they are required, ROS levels must be kept in check by the glutathione-dependent antioxidant machinery (70, 71). Indeed, preventing glutathione (GSH) production impairs

T-cell activation, as the energy and anabolic demands of these cells can no longer be met (72). GSH deficiency alters mammalian target of rapamycin (mTOR) and Myc activation, preventing the metabolic switch to glycolysis and glutaminolysis in an adenosine monophosphate–activated protein kinase (AMPK)-dependent manner (62, 73). Paradoxically, it was reported that ROS can also downregulate T-cell activation by regulating the degradation of signaling molecules and the activation of cytoskeletal proteins (74, 75). To prevent excessive ROS from triggering the mitochondrial permeability transition pore (PTP) opening and causing cell death, CD4 T cells upregulate microRNA (miR)-23a, which targets peptidylprolyl isomerase F (PPIF or Cyclophilin D), a key regulator of the PTP. The reduction in PPIF is expected to keep the mitochondrial PTP closed and reduce the escape of ROS, preserving CD4+ T-cell survival during the early hypermetabolic and inflammatory state of the activation process (76).

## ROS CONTRIBUTE TO BCR SIGNALING

Unlike that of T cells, B-cell metabolism is less well characterized. However, it was recently demonstrated that energy demand is elevated during antigen (Ag)-driven proliferation and differentiation (77, 78). B-cell stimulation with lipopolysaccharide (LPS) or anti-immunoglobulin M (IgM) antibodies drastically increases glucose import, although increased mitochondrial respiration still occurs, suggesting that here again mitochondrial function is important (**Figure 1**) (79, 80). Interestingly, ROS production in response to BCR stimulation occurs in two waves. An early NADPH oxidase 2-dependent ROS increase take place within minutes of BCR stimulation, and a second wave of increasing ROS levels from the mitochondria occurs at later time point (81). B cells deficient in early Nox2-dependent ROS production have no defects in proximal BCR signaling, cell activation or the ability to mount an antibody response following T cell-dependent Ag stimulation (81). However, preventing the later ROS increase attenuates BCR-dependent signaling, leading to defective activation, proliferation and response to BCR stimulation (81). These results indicate that the continuous production of mitochondrial ROS at later times during the activation process is critical for BCR signaling and optimal Ag-induced B-cell activation and proliferation, in agreement with findings from gene set enrichment analysis showing upregulation of OXPHOS and the TCA cycle in activated B cells (79, 81).

Lymphocyte activation clearly requires a metabolic reprograming for a diversification of the source of energy and biosynthetic building blocks (62, 82, 83). Therefore, one could wonder whether the observed increase in ROS during cell activation could be a consequence of this metabolic reprograming and reciprocally any genetic manipulation of the ROS input could also alter the metabolism of these cells, questioning the real significance of the ROS in the activation process? This latter possibility is readily excluded by the fact that exogenous $H_2O_2$ can rescue lymphocyte activation in the context of genetic ablation of complex III (61, 66, 79, 81). Taken together, these results clearly demonstrate that cell intrinsic ROS signaling participates in the activation processes of both B and T lymphocytes.

## ROS AND LYMPHOCYTE VIABILITY

We have seen that one of the proximal events following TCR signaling is an increase in ROS production both in the form of $O_2^-$ and $H_2O_2$. One direct consequence of the increase in these ROS in the context of T cell blasts is the initiation of activation-induced cell death (AICD) following the induction of FasL expression (84, 85). In fact, downstream of the TCR engagement, activated ZAP70 phosphorylates liker of activated T cells (LAT), which docks phospholipase Cγ1 that generates inositol 3 phosphate (IP3) and diacylglycerol (DAG) (84). DAG activates protein kinase $C_\theta$ ($PKC_\theta$) and its translocation into the mitochondria to enhance the production of ROS in a mitochondrial complex I-dependent manner, which is necessary for the expression of the ligand of the death receptor Fas (FasL) (84). FasL engages Fas receptor and triggers apoptotic cell death, a process where mitochondrial ROS further play a role, as it was later shown that caspase 3 can induced a ROS-dependent cell death by cleaving the respiratory chain complex I subunit NDUFS1 (30). We have also shown that ROS potentiate the apoptotic cascade by amplifying the release of apoptogenic factor from the mitochondria and increasing oligonucleosomal DNA fragmentation (86). Moreover, exposure to exogenous $H_2O_2$ differentially affects T-cell viability, according to their subset and maturation status. Central memory and effector memory T cells are more sensitive to $H_2O_2$ followed by naïve T cells, among which the CD8+ effector memory T-cell compartment is more sensitive to even low doses of $H_2O_2$ (**Figure 2**) (87, 88). In this context, exogenous $H_2O_2$ exposure triggers cell death in a mitochondrial pathway-dependent manner (87, 89). T cells treated with $H_2O_2$ experience the opening of the mitochondrial permeability transition pore (PTP), a rapid decrease in the mitochondrial transmembrane potential $\Delta\Psi m$, and the release of cytochrome C (89). Blocking the mitochondrial PTP opening or interference with the respiratory electron transport chain with rotenone or menadione abrogated $H_2O_2$ cytotoxicity (89). Interestingly, antimycin A, a respiratory chain complex III inhibitor that increases the release of mitochondrial ROS, enhanced apoptosis, while overexpression of Bcl-2 and the viral anti-apoptotic proteins BHRF-1 and E1B 19K counteracted $H_2O_2$-induced T-cell apoptosis (89). Furthermore, inhibition of the transcription factor NF-κB protected cells from $H_2O_2$-induced cell death in a process that likely relies on the expression of a death effector gene such as p53 (89). Paradoxically, T regulatory cells, which have lower intracellular ROS levels, are particularly protected from $H_2O_2$-dependent inhibition of suppressive function and $H_2O_2$-induced death (90). Taken together, the higher sensitivity of effector memory CD8 T cells combined with the reduced susceptibility of T regulatory cells to $H_2O_2$-induced death suggest that the oxidized tumor microenvironment (TME) may be a particularly inhospitable site for CD8 T cells and detrimental to T cell-based adoptive cell transfer therapies. This is even more critical as effector memory T cells are the primary phenotype of cells administered during such therapeutic protocols. Thus, research is needed to determine the effect of the TME of chimeric antigen receptor

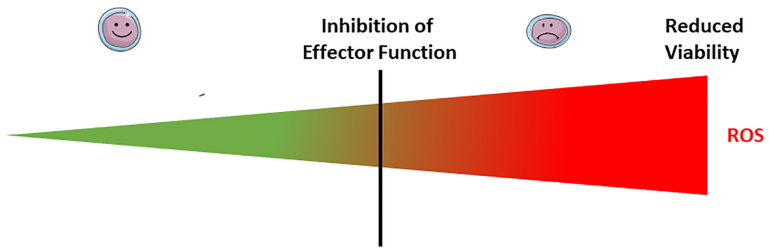


**FIGURE 2** | Exogenous ROS modulate lymphocyte effector functions and viability. At low doses of microenvironmental ROS, both B and T lymphocytes have normal effector function (smiley face lymphocytes). Exposure to mild doses of exogenous ROS affects the lymphocyte effector functions (weary face lymphocytes), while acute exposure to high doses affects their viability. The threshold between mild and acute exposure strongly depends upon the lymphocyte subset and maturation status.

(CAR)-T cell therapies. Beyond apoptosis, ROS critically regulate T-cell viability through the induction of ferroptosis. Both Ag-specific CD8+ and CD4+ T cells deficient for glutathione peroxidase 4 (Gpx4) are unable to expand or protect against viral and parasitic infection (91). This phenotype can be rescued by dietary vitamin E supplementation, indicating that lipid peroxidation-dependent ferroptosis plays a critical role in the T-cell depletion during these antigenic challenges (91).

Exposure of B cells to relatively massive doses (100–250 μM) of $H_2O_2$ has little effect on cell viability in the short term (30 minutes). However, 5 days later, half of the cells have died, even in the presence of CD40 stimulation (92). These doses of $H_2O_2$ although massive are still in the physiological range, as it was estimated that in the proximity of activated neutrophils and macrophages the $H_2O_2$ concentration can reach the 100s of μM (93–95). Moreover, this exogenous $H_2O_2$ exposure completely suppresses the ability to CD40 stimulation to trigger antibody production (**Figure 2**) (92). In fact, exogenous $H_2O_2$ exposure dissociates TRAF2 from CD40, leading to inefficient IKK phosphorylation, IκBα degradation, and NF-κB activation, which altogether severely compromises B-cell activation (92).

## ROS-MEDIATED LYMPHOCYTE DYSFUNCTION

As serial killers, cytotoxic T lymphocytes and NK cells must recognize, engage, kill, and detach from the first target cell before moving to the next (96–98). Detachment of the effector cell from its target requires its repolarization through the reorganization of its cytoskeleton for the disassembly of the first immunological synapse (99). Effector killer cells are quite sensitive to the redox status of their immediate environment (100–102). Oxidizing reagents curb killer cell degranulation, consequently inhibiting their cytotoxicity (102, 103). Interestingly, it was reported that oxidized low-density lipoprotein (ox-LDL), used as oxidizing agent, inhibits killer cell degranulation (102). Pretreatment of NK cells or co-incubation of NK/target cell conjugates with non-cytotoxic doses of ox-LDL markedly and significantly reduces the NK cytotoxic activity against U937 tumor cells (102). This reduced NK cell cytotoxicity is not the consequence of their inability to engage the target cells, because the number of NK:target cell conjugates was not affected nor were the expression levels of CD11a, CD11b, CD18, CD2, and CD62L, key adhesion molecules involved in the effector–target cell interaction (102). Mechanistically, ox-LDL triggers a partial depolarization of the microtubule network that is critical for the polarization of the cytotoxic granules toward the immunological synapse formed between the effector and target cells. Similarly, exposure of mitogen-stimulated peripheral blood mononuclear cells (PBMCs) to ox-LDL reduces their production of TNF-α, IFN-γ and IL-12 (102). Likewise, exogenous and endogenous nitric oxide (NO) inhibits degranulation of lymphokine activated killer (LAK) cells (103). NO inhibits LAK cell exocytosis in part by decreasing the expression of RAS, a critical component of the exocytic signaling cascade, following destabilization of RAS mRNA (103). NO acts by interfering with the mRNA-stabilizing factor HuR, which binds and stabilizes AU-rich elements of the mRNA 3'-untranslated region (104). It was further demonstrated that ROS induced oxidation of the C-terminal portion of the TCRζ chain, and the membrane proximal domain of p56(lck) and cofilin promote their degradation or inactivation, suggesting that ROS can also curb the TCR signaling cascade (74, 75). Similarly, increasing evidence suggests that the dynamics of the immunological synapse can be regulated by ROS through their direct or indirect effects *via* plasma membrane polarization on calcium signaling and effector cell cytoskeletal reorganization (99, 102, 105–107). As stated earlier, activation of the lymphocytes after engagement of their receptor initiates a phosphorylation cascade, resulting in, among other things, the mobilization of intracellular $Ca^{2+}$ stores, which is essential for the gene expression crucial for lymphocyte activation and the development of adaptive immunity (99, 108–111). Depletion of $Ca^{2+}$ stored in the endoplasmic reticulum triggers store-operated $Ca^{2+}$ entry (SOCE). Compared to Orai1, the $Ca^{2+}$ channel involved in SOCE, Orai3 lacks the redox-sensitive cysteine 195 and therefore is redox-insensitive. Co-expression of Orail3 with Orai1 reduces SOCE sensitivity to ROS inhibition. Consequently, it is not surprising that T lymphocytes display upregulated Orai3 expression during their differentiation into effector T cells. This means that the modulation of the Orai1:Orai3 ratio could be a possible mechanism by which effector T lymphocytes preserve some responsiveness in oxidized environments, such as the hypoxic TME or inflamed tissues (106, 112).

ROS also regulate the effector function of B cells. Overexpression of a phosphorylation-defective mutant of succinate dehydrogenase A to model excessive mitochondrial ROS production suppresses Ig production, germinal center (GC) formation, and GC B-cell proliferation following an encounter with T cell-dependent Ag. Excessive mitochondrial ROS production also suppresses Ig production against T cell-independent Ag (113) as well as BCR-dependent Lyn, Btk, and PLCγ2 phosphorylation and CD19 expression. From these collective results, it was hypothesized that excessive mitochondrial ROS dampen B-cell activation most likely by reducing CD19 expression (113). Overall, it seems that mild to moderate exposure to exogenous ROS affects the lymphocyte effector functions, while acute exposure affects their viability (**Figure 2**). The situation is complicated by the fact that the threshold between mild, moderate and acute exposure strongly depends upon the lymphocyte subset and maturation status.

## ROS IN IMMUNE CELL DYSFUNCTION: THE CASE OF AUTOIMMUNITY

As we have discussed earlier, endogenous ROS contribute to lymphocyte activation; however, depending of the lymphocyte activation and/or differentiation status, exogenous ROS can affect their effector function and viability. Thus, we also would like to discuss whether ROS could play a role in the pathogenesis of immune-related disorders such as autoimmunity where the wrath of the immune response mistargets self-antigens (autoantigens). For instance, abnormal functions of T helper (Th)-17 cells, which in a normal setting are essential to fight against extracellular bacteria (114–116), are involved in multiple chronic inflammatory disorders such as psoriasis, multiple sclerosis (MS), inflammatory bowel disease (IBD), Sjögren's syndrome and rheumatoid arthritis (114). Interestingly, using sublethal doses of oligomycin A, an inhibitor of the respiratory chain ATP synthase/complex V, it was shown that mitochondrial oxidative phosphorylation (OXPHOS) plays a pivotal role in for Th17-mediated autoimmunity (117). Oligomycin treatment abolished Th17 pathogenicity by altering the expression of Th17 pathogenic signature genes, such as transforming growth factor beta 3 (TGFβ3), interleukin 23 receptor (IL-23R), signal transducer and activator of transcription 4 (Stat4), and G protein-coupled receptor 65 (Gpr65), while genes inversely associated with Th17 pathogenicity such as suppressor of cytokine signaling 3 (Socs3) and IL-10R subunit alpha (IL-10Ra) were upregulated (117). Although the authors did not directly test this possibility, it is very likely that mitochondrial ROS could be involved in this process, as in their experimental condition the oligomycin treatment severely suppresses the basal mitochondrial oxygen consumption rate, which could result in a severe reduction in mitochondrial ROS, indicating that ROS could protect against the pathogenicity of Th17 cells (117). This agrees with previously work by Tse and coworkers showing that prevention of $O_2^-$ production by macrophages and T cells skews T-cell polarization toward Th17 (118). Although they used a model of NOX-deficiency, collectively these results agree that the absence of ROS alters T-cell lineage commitment, pointing to a role for superoxide in the modulation Th17 *versus* Th1 T cell responses (118). From a more clinical stand point, it was shown that the hypomorphic allele of the Ncf1 gene encoding for $p47^{phox}$, a subunit of NOX2, is one of the strongest genetic predispositions for autoimmune arthritis, autoimmune encephalomyelitis and systemic lupus erythematosus (SLE), which are associated with increased numbers of autoreactive T cells (119–122). Interestingly, results from two clinical trials, one using N acetyl cysteine the other Sirolimus to modulate respectively cellular GSH content and mTOR activity resulted in the improvement of SLE condition suggesting that modulation of the mitochondrial ROS output could also contribute to regulate pro inflammatory T cell development (123, 124). Furthermore, by downmodulating the efficacy of antigen processing, ROS may further contribute to limiting the activation of autoreactive lymphocytes. In this regard, in the early stage of the processes, ROS should not simply be considered as effectors to eliminate invading pathogens, but also as modulators to fine-tune the inflammatory response depending on the timing, the site and the level of their production (125, 126). By contrast, in the context of dysregulated and prolonged chronic inflammation, the local microenvironment is characterized by a low nutrient levels, increased lactate production, decreased pH, hypoxia and an increase level of ROS, which collectively lead to excessive tissue destruction. At this later stage, this excessive tissue destruction could promote the accessibility to cryptic neoantigens favoring the progression and exacerbation of autoimmunity (126).

## ROS IN THE PROCESS OF CYTOTOXIC LYMPHOCYTE KILLING

Cytotoxic lymphocytes are particularly efficient at eliminating target cancer cells and virally infected cells. They mainly used the cytotoxic granule pathway relying on the degranulation of the pore forming protein perforin and a family of five serine proteases call granzymes in human (127–129). Although the granzymes trigger very distinct cell death pathways, we found that granzyme A and B (GA and GB) share the ability to induce ROS-dependent cell death. It was demonstrated that GA induces ROS-dependent death that is independent of the mitochondrial outer membrane permeabilization (MOMP) and insensitive to BCL2 but has all the morphological features of apoptosis (127, 130–133). We also showed that ROS are necessary for the rapid cell death induction by GB. We found that K562 cells treated with a sublytic concentration of perforin (P) and GB undergo a rapid increase in ROS production and cell death that is inhibited in the presence of the well-characterized antioxidants N-acetyl cysteine (NAC), superoxide scavenger MnTBAP, or the mitochondrial targeted superoxide scavenger MitoQ (134). Moreover, GB and P-induced ROS and cell death are completely absent in pseudo rho cells deficient for mitochondrial DNA (mtDNA) and therefore lacking a functional respiratory chain (134). Both GA and GB induce ROS release from isolated intact mitochondria in the absence of cytoplasmic fraction S100 (130). Using organelle proteomics and bioinformatics, we found that GA and GB cleave NDUFS3, NDUFV1, NDUFS1 and NDUFS2 iron-sulfur (Fe-S) cluster-containing subunits of the respiratory chain complex I (86, 130,

132, 135). Cleavage of complex I subunits exposes iron sulfur clusters and dramatically increases electron leak from the respiratory chain, leading to a rapid and sustained mitocentric ROS production, loss of complex I, II, and III activities, disorganization of the respiratory chain, mitochondrial respiration impairment, and loss of mitochondrial cristae junctions (86, 130, 132, 135, 136). It is worth noting that another study has also suggested the contribution of NOX as source of ROS during GB-mediated cell death (137). However, we found that, GB-mediated killing of mouse embryonic fibroblasts (MEFs) from NOX-deficient animals proceeds as in wild-type MEFs (86). GB induction of mitocentric ROS promotes apoptogenic factor release and oligonucleosomal DNA fragmentation (138, 139). Although granzymes do not express a mitochondrial targeting signal, they enter the mitochondria independently from the TOM40 complex, the organelle entry gate, and use instead the SAM50 channel (136, 140). SAM50 is the core channel of the mitochondrial sorting and assembly machinery dedicated to the insertion of *de novo* β-barrel proteins into the mitochondrial outer membrane (141–144). Preventing the entry of granzymes into the target cell mitochondria alters their cytotoxicity. Using a model of human glioma, a very aggressive primary brain tumor for which there is no cure, we showed that granzyme mitochondrial entry is also essential for the reduction of tumor burden *in vivo* (136, 140). Collectively, these interesting results also indicated that respiratory chain complex I is at the crosstalk of GA, GB and caspase 3, three different cell death pathways. Complex I targeting is also conserved across phylum from bacteria to mammals. In collaboration with the Walch's group we showed GA- and GB-mediated disruption of bacterial complex I is also a necessary step for bacterial death (145). The central role of complex I alteration during cell death suggests that it is a very important step whose full range of function has yet to be unraveled. For more about the antimicrobial action of the granzymes, we refer readers to the review of the oxidative and non-oxidative antimicrobial activities of the granzymes by Marilyne Lavergne on this same research topic.

## CANCER, OXIDATIVE STRESS, AND CYTOTOXIC LYMPHOCYTES

Uncontrolled proliferation and neoplastic transformation come with enormous demands for energy and macromolecule building blocks. These demands impose a severe metabolic stress, requiring a striking reprogramming of the cancer cell metabolism (146, 147). The resulting altered metabolism combined with the hypoxic nature of the TME is accompanied by marked production of ROS (148–151). This overproduction of ROS activates the cellular antioxidant response based on enzymatic and non-enzymatic antioxidant molecules, which is under the transcriptional control of the transcription factor nuclear factor (erythroid-derived 2)-like 2 (NRF2). Three isoforms of superoxide dismutase (SOD), cytosolic CuZn-SOD (SOD1), mitochondrial Mn-SOD (SOD2), and extracellular EC-SOD (SOD3), are involved in the rapid dismutation of $O^{2-}$ into $H_2O_2$ (15, 16). The homotetrameric catalase converts $H_2O_2$ into water using NADPH as a cofactor (15, 17). The glutathione peroxidases (GPx) use glutathione (GSH) and reduce $H_2O_2$ and lipid hydroperoxides (15, 18). $H_2O_2$ removal also involves thioredoxin (TRX), thioredoxin reductase (TRR), thioredoxin peroxidase (PRX) and glutaredoxins (15). The most abundant non-enzymatic antioxidant molecule in the cell is GSH, which participates in the reduction of $H_2O_2$ into $H_2O$ and $O_2$, and is thereby oxidized to form GSSG. GSSG is then recycled into GSH by glutathione reductase still using as electron donor NAD(P)H. GSH also maintains aqueous and lipophilic levels of the antioxidant ascorbic acid (vitamin C) and α-tocopherol (vitamin E), respectively. Nevertheless, when this antioxidant system is overwhelmed, the pro-oxidant/anti-oxidant equilibrium is lost, and a state of oxidative stress is reached where the cells accumulate oxidative damage in all type of macromolecules, including DNA, RNA, lipids and proteins, which could lead to cell death (152–154). Oxidative DNA modifications generate 8-hydroxy-2′-deoxyguanosine, which contributes to the accumulation of mutations that enhance aging and carcinogenesis (155). Consequently, transformed cells adapt and reach new redox balance, and paradoxically, ROS instead of killing, stimulate tumor development and progression by promoting cell proliferation through their mitogenic action as activator of extracellular-regulated kinase 1/2 (ERK1/2). This induces ligand-independent receptor tyrosine kinase (RTK) activation, activating Src kinase, NF-κB and phosphatidylinositol-3 kinase (PI3K)/Akt, to enable evasion of apoptosis and anoikis as well as to induce metalloproteinase (MMP) release in the extracellular matrix to favor invasion and promote angiogenesis (156–161). ROS also contribute to epithelial to mesenchymal transition (EMT), an important process in the metastatic dissemination of cancer cells (2). Importantly, in the TME, cancer cells reprogram other cells, such as cancer-associated fibroblasts (CAFs), endothelial cells and cancer-associated macrophages (CAMs), in a ROS-dependent manner to favor tumor progression. CAFs contribute to tumor growth by promoting the tumor angiogenesis by secreting VEGF and angiopoietin, by generating anti-apoptotic factors and by the secretion of chemokines (CCL2 and CCL5) and MMPs to promote the dissemination while blocking the immune response through the secretion of immunosuppressive cytokines IL-6, IL-10 and TGF-β (162–164). This marked production of ROS also alters the phenotype of innate immune cells infiltrating the tumor parenchyma, contributing to the noxious nature of the TME (165–168). Interestingly, it is worth noting that a direct link exists between the environmental ROS of the TME and inflammation (169). Intracellular ROS may regulate EMT in a NF-κB– and hypoxia-inducible factor 1 (HIF-1α)–dependent manner in a process requiring the activity of cyclooxygenase-2 (COX-2), the first enzyme in the synthesis of prostaglandins, prostacyclin and thromboxanes including prostaglandin E2 (PGE2). This suggests that this oxidized microenvironment favors a state of chronic inflammation in the TME. As stated earlier cytotoxic lymphocytes (NK cells and cytotoxic T lymphocytes) play an essential role in the immune response against cancer (97, 170–177). It is therefore not surprising that harnessing the power of these innate and adaptive cytotoxic immune cells during immune check point blockage (ICB) or CAR-T/NK cell immunotherapies has produced very

encouraging results (178–180). As we have discussed earlier, NK cells and CD8+ effector memory T cells are particularly sensitive to even low doses of $H_2O_2$ while the T regulatory cells are protected from $H_2O_2$-dependent inhibition of their suppressive function and $H_2O_2$-induced death (87, 88, 90). Accordingly, tumor-infiltrating lymphocytes must adapt to this oxidized microenvironment among other things by modulating the ratio ORAI1:ORAI3 expression (106, 112). Despite these adaptation mechanisms, as we have seen earlier, exposure to exogenous ROS can severely dampen lymphocytes' effector function, making the TME particularly hostile to infiltrating lymphocytes (181). Interestingly and counter intuitively, the inflamed nature of the TME further contributes to making the TME hostile for lymphocytes and NK cells. Indeed, it was recently reported that tumor-derived PGE2 achieves immune evasion by inhibiting NK cell-mediated remodeling of the TME and unleashing of cytotoxic T cells (173). Interestingly, F2-isoprostanes (F2-IsoPs) and isolevuglandins (IsoLGs), which are oxidized derivatives of PGE2, are extremely relevant disease biomarkers, as they are directly involved in the pathological processes (induction of inflammatory pathways, modulation of immune response, and induction of cell death) (182–184). Since inflammation is closely linked to ROS production, whether the oxidized form of PGE2 contributes to this immune evasion needs to be investigated. Collectively, the evidence supports that the oxidized nature of the TME is likely to affect the efficiency of infiltrating anti-tumor lymphocytes and the development of strategies to enable lymphocytes to withstand the oxidized nature of the TME could improve immunotherapies (**Figure 3**).

## CONCLUDING REMARKS

Based on the available evidence, both NOX- and mitochondrial-derived ROS play critical roles in lymphocyte activation, development, effector function, cytotoxicity, viability but also dysfunction. To this regard ROS directly contribute to both physiological and pathological adaptive immune responses.

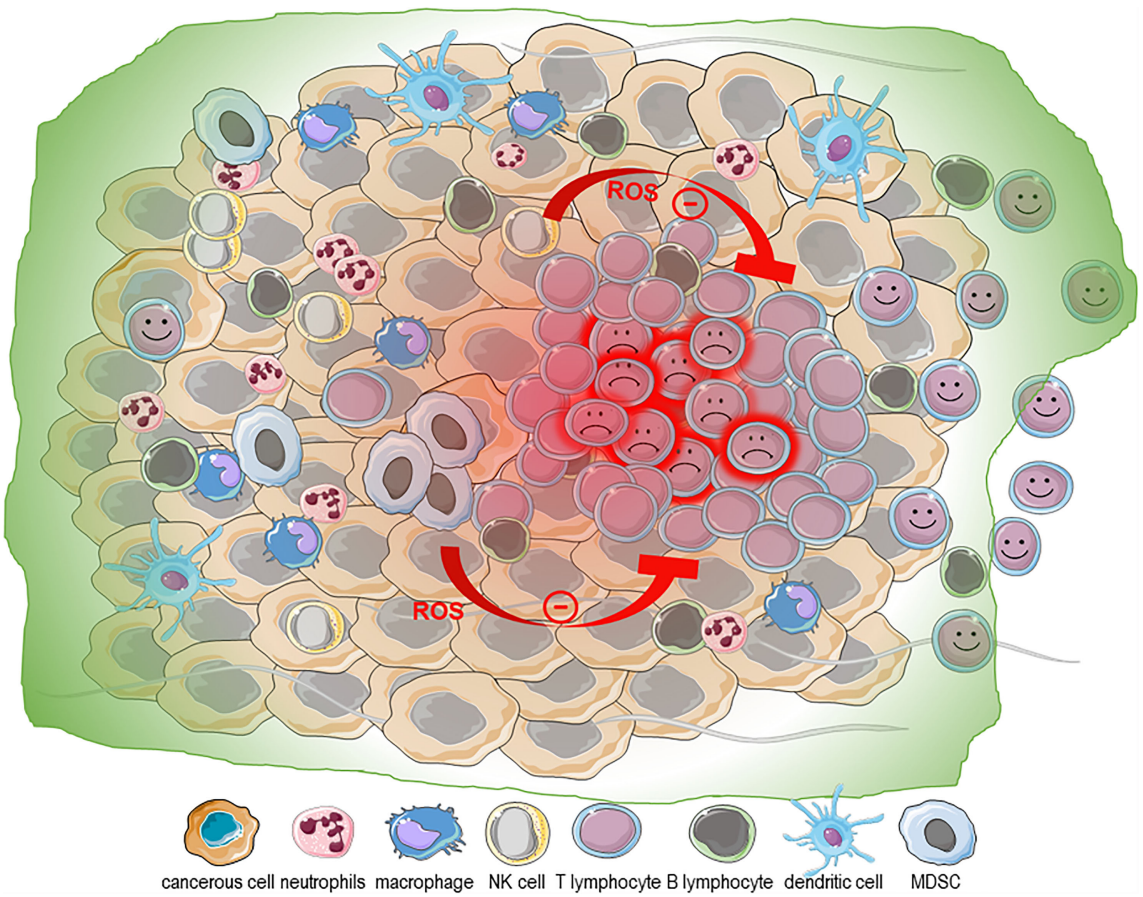


**FIGURE 3** | ROS in the tumor microenvironment. The metabolic stress imposed by the neoplastic transformation and uncontrolled proliferation combined with the hypoxic nature and marked production of ROS contribute to the chronic inflammation state of the tumor microenvironment (TME). The oxidative nature of the TME is exacerbated by its infiltration with immune cells with altered phenotypes [neutrophils, macrophages and myeloid derived suppressor cells [MDSCs)]. NK cells and CD8+ effector memory T cells are particularly sensitive to even low doses of $H_2O_2$, while the T regulatory cells are protected from $H_2O_2$-dependent inhibition of their suppressive function and $H_2O_2$-induced death. Taken together, this supports that the oxidized TME may be a particularly inhospitable site for NK cells and CD8 T cells and detrimental to T cell-based adoptive cell transfer therapies.

ROS from both sources contribute to the activation process of lymphocytes, however, based on strong genetic evidence relying on hypomorphic allele of the Ncf1 gene encoding for $p47^{phox}$, it is tempting to suggest that NOX-derived ROS would have a preponderant role as modulators to fine-tune the inflammatory response depending on the timing, the site and the level of their production. But more investigations are still required to seal this case. Moreover, since ROS also participate in innate cell function by potentiating the killing ability of phagocytes, an essential step in the antigen processing and presentation function of these phagocytes, ROS also indirectly contribute to adaptive immunity though the interplay between innate and adaptive immunity. Further characterization of the complex functions of ROS in lymphocyte biology will bring new insight for understanding the pathological conditions in which lymphocyte function is either detrimental or beneficial.

## AUTHOR CONTRIBUTIONS

DM, EB, and MW wrote and made the illustrations. All authors contributed to the article and approved the submitted version.

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