Nitric oxide and redox mechanisms in the immune response

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Abstract

The role of redox molecules, such as NO and ROS, as key mediators of immunity has recently garnered renewed interest and appreciation. To regulate immune responses, these species trigger the eradication of pathogens on the one hand and modulate immunosuppression during tissue-restoration and wound-healing processes on the other. In the acidic environment of the phagosome, a variety of RNS and ROS is produced, thereby providing a cauldron of redox chemistry, which is the first line in fighting infection. Interestingly, fluctuations in the levels of these same reactive intermediates orchestrate other phases of the immune response. NO activates specific signal transduction pathways in tumor cells, endothelial cells, and monocytes in a concentration-dependent manner. As ROS can react directly with NO-forming RNS, NO bioavailability and therefore, NO response(s) are changed. The NO/ROS balance is also important during Th1 to Th2 transition. In this review, we discuss the chemistry of NO and ROS in the context of antipathogen activity and immune regulation and also discuss similarities and differences between murine and human production of these intermediates.

Keywords: oxidative nitrosative stress, signaling cascade

Introduction

Over the course of the last three decades, the term "oxidative stress" has become a household word. The concept that excess oxidation leads to aging and to many diseases has been thoroughly implanted into the psyches of our culture. Molecules such as ROS, similar to those produced from ionizing radiation, can be

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generated in the body and when uncontrolled, can lead to cellular and tissue damage [1, 2]. Antioxidants have frequently been touted as the antidote to oxidative stress but are only part of the complex chemical story. Although oxidation can lead to toxicity, it is important to realize that redox-based molecules are essential mediators of critical functions in physiological systems and are essential to immunity against disease. In fact, redox reactions are key signal transduction mechanisms and should be included in the pantheon of interwoven cell regulatory systems along with phosphorylation reactions mediated by kinases/phosphatases and other types of cellular signaling pathways [3–5]. Redox molecules such as peroxide, superoxide, NO, and RNS, once thought to be only toxic, are essential in tissue repair [6, 7]. More recently, other toxic gases, such as CO and H_2S, have emerged as therapies to correct dysregulated cell functions [8, 9]. As indicated in **Fig. 1**, there are two major roles for these reactive small molecules in the immune response: direct participation in the eradication of pathogens or regulation of immune pathways. However, it is only NO/RNS and ROS that serve "double duty" by participating as immunotoxins as well as immunomodulators. Not only does the incoming immune signal initiate the production of NO and ROS to intercept and kill pathogens, but downstream signaling pathways that lead to the full expression of the immune response are modulated by NO and ROS in complex ways. Overall, the complexity of these reactions is a reflection of the diverse nature of redox chemistry within a biological setting.

EARLY STUDIES CONNECTING NO AND IMMUNITY

The discovery of NO as an endothelial-derived relaxation factor and as the active ingredient in nitroglycerin (a potent vasodilator) clearly demonstrated that redox-reactive molecules, normally thought to be toxic, were endogenously generated within the cardiovascular system [10–12]. Furthermore, these molecules served important, normal physiological functions [11]. Concurrently, NO was found to be an important component of the immune system. Hibbs and coworkers [13, 14] showed that a substance that was released by macrophages and exhibited antipathogen and antitumor activity also required arginine for its production. These data supported the earlier observation by Tannenbaum and coworkers [15], who showed that plasma levels of nitrite and nitrates increased upon infection, suggesting an increase in endogenous production of NOs. This group went on to show that nitrosoproline was formed in vivo, demonstrating for the first time that not only did nitrosative chemistry occur beyond the gastrointestinal tract but also that humans generated high levels of NOs in response to illness [16, 17]. Hibbs and coworkers [18, 19] further connected immunity and NO when they showed that IL-2-mediated immune activation increased NO levels in patients and promoted tumor eradication in mice. Direct evidence that macrophages made nitrite and nitrate, as well as nitrosamines, was shown in the late 1980s by a number of groups [20–22]. Miwa et al. [23] showed that nitrosocompounds were detected in animals that were treated with LPS. Stuehr and Nathan [24] went on to show that NO generated by macrophages could kill leukemia cells. Finally, Lancaster and Hibbs [25] demonstrated the formation of iron-NO complexes within macrophages. In total, these data provided strong evidence that NO plays a critical role in the immune response and opened the door to understanding the participation of redox molecules and their reactions in immunity and to a greater appreciation of their importance.

NO AND ROS ARE THE PRINCIPLE REDOX MOLECULES IN IMMUNITY

The primary source of NO is the NOS enzyme, which has three isoforms; two are constitutively expressed, and one is inducible. eNOS (*NOS3*) and nNOS (*NOS1*) are constitutively expressed and in general, release short bursts of NO in a calcium-dependent manner [26]. More recent studies have found that post-tran-

scriptional modification of eNOS can convert short, calcium-dependent NO bursts to sustained, calcium-independent production of NO [27]. In the case of eNOS, this post-transcriptional regulation plays a fundamental role in the health of endothelium during angiogenesis, as well as regulation of immune activation. The inducible form of iNOS (*NOS2*) is generally associated with the immune system and produces NO for prolonged periods of time in a calcium-independent manner. Levels of NO produced by iNOS in the microenvironment of the cell can range from as low as 10 nM to µM amounts for days [28]. By producing high levels of NO, iNOS activity can orchestrate varied NO-modulated microenvironments within a tissue, each with potentially different functions. Thus induction of iNOS is not solely defined by high, local amounts of NO, as iNOS activity can generate a wide range of levels of NO for variable periods of time [29]. Thus, iNOS provides unique flexibility in dealing with an immune challenge. In contrast, the low flux of NO produced by eNOS restricts its actions. To be effective, eNOS or nNOS must be targeted and anchored/sequestered in close proximity to its target. An additional level of immune regulation by iNOS is provided by the ability of the NOS enzyme to make products other than NO. These include NOHA and O_2^-. By generating NOHA, an inhibitor of AG activity, the iNOS system can affect pathways that mediate cell growth (ornithine to polyamines) or tissue matrices (ornithine to proline) [30]. This diversity of NOS activities can produce different temporal and concentration profiles of NO as well as other products to facilitate and broaden the functional versatility of these enzymes during immune challenge.

The second basic biological redox system involved with immunity is comprised of O_2^-, H_2O_2, and other ROS, known collectively as ROS. This system can function independently of NO to carry out specific oxidation events that modify intracellular signaling pathways, including regulation of migration [31], cell mitogenic potential [32], or host defense. The primary cellular sources of ROS are oxidases that generate O_2^- by the transfer of a single electron to oxygen from NADPH (reduced form). Further single electron reduction to H_2O_2 or other ROS is catalyzed by a series of enzymes that includes SOD and MPO through interactions with transition metals or through reactions with NO.

The first NOX to be identified as a participant in the innate immune response was found in phagocytic cells. Originally, the activity of this enzyme was described as the "respiratory burst" and was associated with intracellular killing of bacteria or other invaders [33]. It is now known that this mechanism generates a rapid increase and high level of superoxide when phagocytes are exposed to bacteria through a heteromeric assembly of multiple protein components. These components include a membrane-bound cytochrome b558 complex made up of gp91phox and p22phox catalytic subunits and four cytosolic proteins (p47^{phox}, p67^{phox}, p40^{phox}, and GTPase Rac1). During immune stimulation, the cytosolic proteins assemble into a complex and translocate to the membrane, joining with the gp91phox-p22phox complex. This step results in full activation of the enzyme complex and the production of O_2^- [34–36]. gp91phox is also now known as NOX2 and is a member of the *Nox* gene family of NOX that includes homologs of NOX2 (NOX1, NOX3, NOX4, and NOX5) and related DUOXs, termed DUOX1 and DUOX2. NOX1, NOX3–5, and DUOX1 and -2 are most commonly found in nonphagocytic cells, such as smooth muscle or endothelial cells, where they produce limited levels of O_2^- (or H_2O_2 in the case of DUOX1 and -2) in a tightly regulated, temporal-spatial pattern [31].

Activation of the NOX is initiated by the interaction of immunogens with specific membrane receptors such as complement or FcRs. NOX is also stimulated by treatment with agents that activate calcium entry or by treatment with cellular products such as arachidonic acid [34–36]. Additional exposure to "priming" agents such as IFN-γ, TNF-α, or IL-1β significantly increases the levels of O_2^- produced during the im-

mune response. In some cases, low levels of superoxide and H_2O_2 are constitutively produced, primarily by NOX4 or DUOX activation [37, 38]. Although the activation process has been well described, less is known about the mechanisms that limit NADPH-oxidase activity and superoxide production or help to tightly regulate its outcomes. Potential deactivation mechanisms range from dephosphorylation of complex components by phosphatases to membrane depolarization or loss of charge counterbalance mechanisms (e.g., loss of proton or chloride channels associated with oxidase activity) and loss of PLA2 and arachidonic acid production [35, 39–44]. Many of these mechanisms are modulated by NO, where NO plays a key role in controlling levels of ROS and in limiting the reactivity of O_2^- and H_2O_2 to specific cellular sites. Restricted delivery of superoxide under specific conditions and locations helps minimize the collateral damage that may be initiated by ROS. Compartmentalization of superoxide/H_2O_2 production is also used to control signaling processes. For example, endothelial cell migration and endothelial growth factor signaling during processes such as wound repair are dependent on NADPH-oxidase activity [31, 45, 46]. For migration, the NOX complex is located to specific membrane regions associated with directed movement of the cell [31].

REDOX CHEMISTRY OF THE IMMUNE RESPONSE

To understand how NO and redox molecules carry out their diverse immune functions, it is important to understand the type of chemical reactions that produces those outcomes. In general, there are two classes of redox chemistry based on NO or O_2^- as the starting molecular species. The first class involves NO itself and RNS made from the interaction of NO with ROS. These are frequently called RNS. The second class involves ROS as the starting molecule species. NO/RNS and ROS can be used separately or in combination in immune responses and participate in "killing" and immune regulation. As discussed above, NO is produced as a primary product of the enzymatic action of iNOS [47]. NO is relatively nonreactive and only reacts directly with transition metals in heme or cobalamine, with nonheme iron [48], or with reactive radicals such as hydroperoxide radicals formed during lipid peroxidation [48]. This latter reaction exemplifies the potent antioxidant capability of NO. It is now well established that NO acts directly as a powerful antioxidant in mammalian cells and prevents ROS injury initiated by reactions with metals, with superoxide, or with lipid radicals [49]. In contrast, NO does not react with thiols or other nucleophiles directly but requires activation with superoxide or O_2^- to generate RNS such as $ONOO^-$, NO_2, and N_2O_3 [48]. $ONOO^-$ and NO_2 can oxidize substrate, and N_2O_3 is a primary source of nitrosation of substrate. Thus, these RNS participate in the generation of conditions leading to nitrosative or oxidative stress [48].

There are several key chemical concepts that are important for understanding the role of RNS and ROS intermediates in vivo. The first is that NO and NO_2 are lipophilic, and both can migrate through cells, broadening the potential profile of targets and reactivity. As a RNS, $ONOO^-$ reacts rapidly with CO_2 to form CO_2-$OONO^-$, which shortens its lifetime to <10 ms [50]. The anionic character and short lifetime drastically limit its mobility, thereby effectively prohibiting migration across membranes. As a result of the short lifetime, the location of superoxide formation dictates the location of $ONOO^-$ formation. When NO levels are higher than superoxide levels, the CO_2-$OONO^-$ intermediate is converted to NO_2 and N_2O_3 and changes the redox profile from an oxidative to a nitrosative microenvironment [51]. The balance between NO and superoxide then determines not only the bioavailability of NO but also can generate RNS in the close proximity of a superoxide source, consequently producing a different reaction profile. Another important consequence of ROS and NO interaction is that peroxidase activity leads to consumption of NO and

the formation of nitrite [52]. The same peroxidase and Fenton reactions that consume NO can also oxidize nitrite to NO_2 to increase oxidation and nitration further [28]. Thus, ROS consumes NO to generate NO_2 and N_2O_3, as well as nitrite, in specific locations.

REDOX CHEMISTRY OF THE IMMUNE RESPONSE: ROLES IN ERADICATION OF BACTERIA, PARASITES, AND VIRUSES

The phagosome "cauldron"

Studies on bacterial killing by the NOX clearly show the compartmentalization of ROS production to phagosomes. Delivery of superoxide under specific conditions and at specific locations helps to minimize the collateral damage that may be initiated by ROS to other subcellular organelles within close vicinity of the phagosome. However, the phagosome is a uniquely restricted space (**Fig. 2**). As NOX2 is a plasma membrane protein, which upon uptake of pathogens, directs O_2^- release into the phagosome, it is likely that any oxidizing species produced by NOX2 is restricted to the phagosome interior. In contrast, iNOS is not commonly, spatially restricted, and the readily diffusible nature of NO and RNS allows these redox species to participate in pathogen killing in a widespread and in an interesting concentration-dependent manner. The level of NO within phagosomes will depend on the specific NOS isoform as well as the level of NOS activity and thus, can be varied by a number of cellular conditions. The presence of NOX and superoxide in the phagosome combines to scavenge NO, resulting in increased nitrite within the phagosome. Nitrite under acidic conditions also contributes to the cauldron effect. Like NO, under specific conditions, nitrite can generate N_2O_3 and NO_2/NO similar to the NO/superoxide and NO/O_2^- reactions described previously. This is particularly important during phagocytosis of pathogens. As phagosomes have an acidic environment, these conditions are compatible with the generation of NO, NO_2, and/or N_2O_3 [53, 54]. Thus, the phagosome may be thought of as a cauldron of redox reactions, which uses RNS and ROS to kill pathogens.

Bacteria

The variety of chemical species generated from NO/RNS and ROS provides an arsenal that can be used by the immune system to combat pathogens. Rather than killing by nitrosative and oxidative modification of critical bacterial macromolecules as once thought, NO and the formation of RNS provide a multifaceted pathogen eradication program [55, 56]. The requirement for the combination of RNS and ROS, rather than NO or superoxide/H_2O_2 by themselves, is shown in the response to *Escherichia coli* [57]. Little bactericidal activity against *E. coli* is observed with bolus treatment of NO or of H_2O_2 [57]. However, when combined together, H_2O_2 plus NO mediate a dramatic, three-log increase in cytotoxicity, as opposed to 50% killing by NO alone or H_2O_2 alone. This indicates that although *E. coli* are resistant to NO/RNS and H_2O_2 separately, these bacteria are highly susceptible to their synergistic actions. The mixture of NO/RNS and ROS effectively provides a potent killing combination that increases bactericidal activity and protects the host cell from ROS-mediated toxicity by the antioxidant and other protective actions of NO. Interestingly, the resistance of *E. coli* to NO/RNS or ROS alone is consistent with studies that show *E. coli* can increase the O_2^- stress regulon, a key gene regulator for the adaptive/antioxidant response to H_2O_2 and superoxide [58].

The synergistic redox action, in this case, acts as a "one-two" punch. Unlike mammalian systems, bacteria use iron sulfur clusters in electron transport. These clusters are located in the periplasmic space and are particularly susceptible to NO's attack. The result is the release of iron into the interior of the cell where is binds to DNA [57]. Unlike mammalian cells, NO does not penetrate easily to the interior of the bacteria and thus, peroxide, via Fenton chemistry, is required to interact with the bound iron. This process oxidizes DNA and leads to its cleavage. In contrast, NO can diffuse to most parts of mammalian cells, where it serves as an antioxidant [59]. These two basic differences make NO/H_2O_2 a perfect killing combination for *E. coli* and at the same time, protects mammalian cells from ROS-mediated toxicity.

Other bacterial pathogens such as *Staphylococcus* exhibit a different response to redox-mediated attack. The simultaneous presence of NO and peroxide in staphylococcal infections leads to a protective effect, where NO reduces ROS-mediated toxicity to the bacteria [60]. Yet, when *Staphylococcus* bacteria are exposed first to peroxide followed by NO, increased toxicity is observed [60]. In this case, it is the sequential exposure to a burst of superoxide/ROS production followed by NO that is critical for the maximum killing effect by these redox molecules. This combination is extremely effective in eradicating *Staphylococcus* bacteria.

The NO/RNS and ROS redox arsenal, however, does not kill all bacteria. For example, the gram-positive bacteria, *Listeria*, is resistant to redox-mediated killing, most likely because of adaptive mechanisms developed by their propagation under harsh environmental conditions [61]. Regardless, high NO levels lead to cytostasis and imprisonment of the bacteria within the infected cell. Nitrosation of the thiol in the pore-forming protein listeriolysin O (which is a cysteine protease) prevents the bacteria from exiting the cells it has infected [61]. As a consequence, the infectivity of the bacteria is reduced. In addition, imprisonment in the cytoplasm allows other immune constituents to eradicate the infection. Thus, for *Listeria*, although resolution of the infection is slow, generation of NO by NOS remains an important event [62–65].

Another bacterium that is susceptible to RNS is *Mycobacterium tuberculosis*. Like other bacterium, *M. tuberculosis* is taken up by lung alveolar macrophages. These bacterium are sensitive to NO and RNS, but in this case, NO_2 is the toxic species. Although μM $ONOO^-$ or 90 ppm NO are toxic, NO_2 at <1 ppm exposure is considerably more toxic [66, 67]. As discussed above, the macrophage can use various conditions to generate NO_2 in the phagosome (Fig. 2). Where anions such as $ONOO^-/CO_2OONO^-$ cannot diffuse through the cell wall, NO and NO_2 will penetrate. Thus, the acidification of the phagosome in the presence of nitrite and NO provides an optimal condition(s) to generate the RNS and ROS required to kill *M. tuberculosis*. Interestingly, oxidation of methionine groups is a major factor in eradication of this pathogen, and *M. tuberculosis* is protected by the activity of methionine sulfoxide reductase. This finding is confirmed by studies showing that *M. tuberculosis* lacking the reductase are killed more readily [68]. Another role of macrophage-derived NO is that it prevents *M. tuberculosis*-induced apoptosis of T cells, thus aiding in the clearance of the disease via adaptive immune mechanisms [69, 70]. Like Listeria, the eradication of tuberculosis in murine models requires iNOS, demonstrating that the chemistry of RNS provides a tool for an aggressive defense [71]. In the phagosome, nitrosative and oxidative conditions exist that are functionally hypoxic, carbohydrate-poor, and capable of perturbing the cell envelope of the pathogen [72]. The ROS is delivered in the first 3 h, followed by FA from 0 to 48 h and then NO from 4 to 72 h [73]. Iron depletion of the organism forces *M. tuberculosis* to undergo anaerobic metabolism, which retards growth. Bacteria also evade macrophage killing by neutralizing reactive nitrogen intermediates [74]. With respect to ROS, it has been shown that hypochlorous acid/hypobromous acid are more effective in eradicating *M. tuberculosis*,

supporting the additional and critical contribution of peroxidases to the eradication of this pathogen [73]. In the presence of nitrite, these peroxidases can also enhance NO_2 formation. Thus, a phagosome microenvironment consisting of ROS combined with acidic nitrite generates $NO_2/N_2O_3/NO$, which is essential for pathogen eradication by the alveolar macrophage. Overall, NO has a dual function; it participates directly in killing an organism, and/or it disarms a pathway used by that organism to elude other immune responses. This illustrates the requirement for redundancy of redox mechanisms that can generate ROS, NO_2, and RNS in bactericidal mechanisms.

Protozoan/parasites

Many common human parasites have been shown to elicit host iNOS induction and the subsequent initiation of immune mechanisms, resulting in the expulsion of the parasite. The list includes species such as *Plasmodia* (malaria), *Leishmania* (leishmaniasis), and *Toxoplasma* (toxoplasmosis), which are responsible for infections in many parts of the world. Among these parasitic, human infestations, malaria is one of the most prevalent. It is also an example of a parasitic infestation that activates host responses regulated by the innate and adaptive immune systems. Studies have shown that severe cases of human malaria are accompanied by increased iNOS activity and the subsequent production of NO [75, 76]. Increased levels of NO were found to be beneficial, as NO was shown to kill parasites [77, 78]. However, it is also interesting to note that elevated NO levels in the blood of malaria patients correlated with reduced parasitic adhesion to endothelial cells, maintenance of microvascular perfusion, and reduced deleterious cytokine production [79]. Kun, Weinberg, and colleagues [80] demonstrated that the increased NO levels and the resulting protection against malaria were linked to a single nucleotide polymorphism in the promoter of the NOS2 gene, termed the NOS2^{Lambaréné} (G-954C) mutation. The exact impact that *Plasmodium falciparum* components have on NOS2 and NOS metabolite production in human monocytes remains unclear, as neither of these redox species was induced by hemozoin, a malarial pigment that is known to act as an immunogen. As discussed in a later section, the lack of NOS2 induction, despite simultaneous treatment of hemozoin with IFN-γ or a cytokine-LPS mix, is typical of human monocytes [81]. On the other hand, mouse monocytes responded strongly to this stimulus by producing significant quantities of NO and NOS metabolites. These results highlight the species-specific differences between human and mouse redox systems.

Plasmodium berghei is a similar malarial species that affects rodents, where ookinetes are susceptible to NO-induced, apoptosis-like programmed cell death while still in the gut of the mosquito [82]. Studies showed that ingested blood and mosquito tissues were the likely sources of NO and postulated that manipulating NO levels in the mosquito's gut could provide a method for controlling *Plasmodium* transmission [83]. Interestingly, the induction of TGF-β under these nitrosative stress conditions leads to down-regulation of the NO defense system [84], suggesting a potential counter-control of malaria in the mosquito.

Leishmania is a successful intracellular parasite that resides in mammalian macrophages. *Leishmania* infestation is controlled by NO produced by iNOS. This response is particularly strong in murine models of infection. A critical aspect of NO metabolism is that NOHA inhibits AG activity, thereby limiting the growth of parasites and bacteria including *Leishmania*, *Trypanosoma*, *Schistosoma*, *Helicobacter*, *Mycobacterium*, and *Salmonella*, and is distinct from the effects of RNS [85]. Therefore, NOHA is decoupled from iNOS catalysis and adds another component of the iNOS arsenal to limit bacterial and parasitic infection [86]. Type 1 and type 2 responses control the availability of arginine, from which NO is generated. The type 1 response increases conversion of arginine to NO by iNOS, and the type 2 response promotes

AG induction [87]. Cytokines and ROS production are also triggered by a LPG produced by the *Leishmania* species [88]. Soluble and membrane LPG, obtained from *Leishmania major* stationary pro-mastigotes, stimulated ROS, IL-10, and IL-12 production in human PMBCs. A subsequent study also used human PBMCs and showed that ROS and cytokine responses to *L. major* LPG involve TLR2, and TLR2-mediated response could be blocked using anti-TLR2 mAb, which also successfully blocked the production of ROS [89]. One class of *Leishmania* chemotherapeutics, pentavalent antimony complexes, such as sodium stibogluconate and SAG, is used to treat visceral *leishmanias*, which appear to kill the parasite through initial increases in the production of ROS, followed by NO. SAG was shown to induce ERK-P via PI3K activation, which leads to the production of ROS. NO production was induced through the effect of TNF-α [90, 91].

Toxoplasma gondii is another example of a human intracellular protozoan parasite that elicits NO-mediated responses. Its infestation is globally disseminated, affecting approximately one-third of the world's population. As with most intracellular parasites, during infestation, the attacking organism is encapsulated in a vacuole. In this case, a parasitophorous vacuole is generated when the protozoan attaches to and incorporates into the host cell [92]. Studies have demonstrated increased infectivity and severity of inflammatory lesions in the CNS of iNOS knockout mice when compared with WT animals. These results suggest that iNOS limits the progression of the disease to the CNS and that the lack of iNOS is detrimental to the host who is unable to control *T. gondii* replication [93].

Virus

NO is also a critical component in the eradication of viruses. Viral replication is inhibited by the induction of iNOS and the subsequent production of NO (HIV-1, coxsackievirus, influenza A and B, rhino virus, CMV, vaccinia virus, ectromelia virus, human herpesvirus-1, and human parainfluenza virus type 3) [94]. Coxsackievirus and CMV murine models show that iNOS reduces viral burden and decreases latency [95, 96]. Similarly, replication of viral influenza may also be reduced by NO; however, the symptoms may increase, suggesting a balance between an antipathogen response versus healing and resolution responses [97]. A number of viruses have been shown to increase iNOS through activation of TLR3 that increases iNOS/COX2. Interestingly, PGE2 has been shown to inhibit clearance of many viruses as a result of its immunosuppressive nature, and increased cAMP enhances viral replication [98]. In contrast, PGE2 inhibits HIV replication in infected macrophages [99].

Also, during viral clearance, elevated NO concentrations can have unwanted consequences. Influenza studies have demonstrated protection against consolidating pneumonitis in iNOS knockout mice [97]. Elevated NO has been shown in humans infected with Dengue fever. The induction of iNOS leads to retarded growth in human monocytes [100]. NO donors have been shown to inhibit the virus replication [101]. However, systemic production of NO can lead to pathological hemorrhagic fever, which is caused by increased leakiness and inhibition of platelet aggregation [102]. As discussed below, NO may play an important role in the shift from a Th1 to Th2 response but also releases a cytokine storm that participates in shock. Thus, NO is a positive and a negative factor in infectious disease [103], which poses a common problem that must be considered during therapeutic development of treatments for these diseases.

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One of the main mechanisms as to how NO participates in viral eradication involves the nitrosation of critical cysteines within key proteins required for viral infection, transcription, and maturation stages. For example, viral proteases or even the host caspases that contain cysteines in their active site are involved in the maturation of the virus [104–107]. In coxsackievirus, two cysteine proteases that cleave viral polyproteins are sensitive to nitrosative-mediated inhibition, which prevents maturation of the virus [108, 109]. HIV-1 infectivity factor can be nitrosated. Reverse transcriptase can also be inhibited by nitrosation, and nitrosation of a low pK_a cysteine residue in the protein limits NF-κB activity, resulting in inhibition of HIV-1 replication [110, 111]. HIV and herpes simplex viruses have critical zinc finger proteins that are also susceptible to nitrosation [112]. Proteins that participate in viral infectivity, such as integrases, can be nitrosated by NO/RNS. The nucleocaspid protein (also a redox-sensitive zinc finger containing Cys_3His) is also susceptible to nitrosation. When nitrosated, this protein cannot bind to DNA and carry out its function as a topoisomerase [113]. The modification of these proteins prevents viral integration into the host DNA. NO increases host CD44 cell surface expression, where HIV-1 can bind and become internalized, suggesting that increasing CD44 may provide a viral uptake and clearance mechanism. However, in HIV infection, this process represents a dormant phase of the disease [114]. Thus, the nitrosative stress environment produced by iNOS may serve to protect against some viruses by inhibiting viral infectivity, replication, and maturation. Interestingly, the reduction of intracellular GSH may be critical to the removal of virus by increasing nitrosative stress.

REDOX REGULATION OF IMMUNE FUNCTION

Regulation of the redox immunomodulators—NO/RNS and ROS

Beyond the chemical interactions of NO and ROS that combine to eradicate pathogens, these redox small molecules are effective immunomodulators that regulate cellular metabolism as well as multiple proinflammatory and repair/tissue-restoration pathways. In this section, we will examine some of the interactive intracellular pathways that regulate levels of NO/RNS and ROS that are important to the immune response. Fig. 3 provides a diagrammatic scheme of these interactive pathways. As might be expected, NOS is a nodal point with multiple inputs and outputs. Regulation of the enzymatic activity of NOS is clearly critical to the production of NO. The cellular supply of cofactors for iNOS activity, such as availability of arginine, BH_4, NADPH, and superoxide, is required for NO production. However, in the absence of arginine or BH_4, uncoupled NOS becomes a O_2^-/H_2O_2 generator [115, 116]. Although originally described for eNOS and nNOS, the flavin-binding sites of the reductase domain of iNOS are a source of O_2^- generation in the absence of arginine [117]. Therefore, metabolic pathways that control arginine and BH_4 play a role in determining the NO/superoxide balance.

Cellular arginine levels depend on multiple factors, including the type of uptake mechanisms found in cells that provide arginine to various intracellular pools and the enzymatic systems that use this semiessential amino acid. Selective arginine uptake occurs via the activity of cationic amino acid (y+) transporters, also known as the SLC7A family of solute transporters, although other, less-selective uptake transporters have also been described. As shown in Fig. 3, arginine is the sole substrate for NOS, but it is also the sole substrate of AG, another key enzyme involved in immunoregulation. Two isoforms of AG have been found and are located in different intracellular compartments [118, 119]. The *AG1* gene is an inducible cytosolic enzyme whose product is ornithine. In turn, ornithine is the starting point for the production of polyamine synthesis (putrescine, spermidine, and spermine), which is produced via a series of enzymatic reactions

that also requires SAM. Polyamines are critical molecules supporting cell function and regulating diverse cellular processes, including DNA stabilization, ion channel transport, and cell proliferation [120, 121]. By metabolizing methionine, polyamine synthesis impacts the formation of HCys, which in turn, affects the sulfonation pathway and consequently, H_2S and GSH production. The second isoform, the *AG2* gene, is localized to mitochondria, where the enzymatic production of ornithine appears to be directed to the formation of proline and ultimately, collagen [122]. In turn, AG is regulated by NOS and NOX activities. NOHA, a product of NOS, inhibits AG, and O_2^- increases AG activity [86, 123, 124]. Importantly, high AG activity is associated with elevated ROS and low NO fluxes.

As also depicted in Fig. 3, cellular redox balance can be shifted simply by altering the effect of NO on its targets. This is commonly achieved by changing the local concentration of NO. For example, NO antagonizes NOX2 assembly through the activation of PPARγ, which in turn, inhibits expression of the p47 subunit required for NOX2 activation [125]. As a result, O_2^- production is suppressed when NOS activity and NO are high. NO also inhibits COX2 activity, reducing production of ROS by this enzyme [126, 127]. Thus, as NO levels decline, oxidative mechanisms increase. Oxidative and nitrosative stress can also decrease intracellular GSH (reduced form) levels, resulting in a reduced antioxidant capability of the cell. However, loss of GSH leads to the induction of the NRF2/kelch-like ECH-associated protein system with induction of the ARE to increase γ-glutamylsynthase and GSH. This safety mechanism helps to increase GSH synthesis and return antioxidant levels to normal. The ARE promoter also controls HO-1 expression, the enzyme responsible for cellular production of CO, another small redox molecule that has been shown to be immunosuppressive [128–130].

Immune-associated redox pathways regulate other important metabolic cell functions that have the potential for widespread impact on cells, organs, and organisms. For example, methionine is the principle source of methyl groups for a variety of methylation reactions within cells, including those involved in epigenetic modification of DNA [131]. The abundance of methylation equivalents is controlled by the competition between the pathways using methionine. For example, production of polyamines (Fig. 3) is controlled by the "AG to ornithine to polyamine pathway". Polyamines are critical to multiple cell functions, such as DNA stabilization, cell proliferation, and membrane channel activity, all of which are also involved in immune-mediated repair processes. Spermine production consumes methylation equivalents, which can reduce the availability of methionine for methylation of DNA and cell proteins [132]. HCys levels may also be changed and in combination with CBS and in the presence of cysteine will affect the production of GSH via the sulfonation pathway [133]. Interestingly, this same pathway is now known to generate H_2S, the least-studied of the small molecule redox immunomodulators [134, 135]. There is also a direct effect of the methylation reactions on NOS. For example, ADMA, a cleavage product of methylated proteins, is a prominent endogenous inhibitor of NOS and reduces NO levels [119].

NO levels dictate the immune signaling pathway

The ability to change the antagonistic relationship between NO and ROS plays a key role in the orchestration of inflammation and tissue restoration. In this section, we will examine how NO/RNS and ROS actively control innate and adaptive immune signaling. This aspect of redox function has been studied most intensively in cells of myeloid lineage, such as monocytes, macrophages, and neutrophils, where the NO/RNS and ROS that is produced by these cells participate in induction, maintenance, and/or termination

of proinflammatory and anti-inflammatory signaling. Similar to the effect of NO on pathogen eradication, the level of NO and more precisely, the temporal and spatial concentration profiles of NO are key factors in determining immune-mediated processes.

The development and use of reliable NO donors have allowed a careful and quantitative analysis of the relationship between NO/RNS concentration and the regulation of specific immune signaling pathways. Brune and coworkers [136–138] were among the first to show a relationship between increasing steady-state levels of NO and the expression of p53 in murine and human macrophage cell lines. In this case, p53 expression was associated with concentrations of NO that led to apoptosis in the macrophage. Further studies, however, have subsequently identified NO concentration profiles directly related to the expression of other key signaling proteins such as HIF-1α and Akt-P [28, 139, 140]. Stimulation of MCF-7 breast cancer cells, macrophages, or endothelial cells with NO at 10–60 nM increased ERK-P and Akt-P expression and activity, and increased HIF-1α expression was found at levels of approximately 100 nM NO [141, 142]. Fig. 4 provides a synopsis of the biological action of NO over a wide concentration range of NO and shows how this concentration-dependent profile varies with distance from the NO source. NO is highly diffusible, and its concentration will change rapidly, as it is diluted as a result of diffusion. Thus, although concentrations at the point source may reach μM levels, within one cell length (10 μ), this concentration will be reduced up to 1000-fold [143]. Time-specific changes are also observed in NO signaling. For example, NO-mediated ERK-P levels initially increased rapidly on exposure to NO donors and then decreased with continued NO exposure [141]. In contrast, HIF-1α levels remained high as long as NO levels were elevated. These data strongly suggest that the duration of the signaling response after initiation by NO may be an important regulatory factor. The transient nature of some NO signaling leads to the idea that similar to intracellular calcium signaling, NO "waves" are formed and can move through the cell. Importantly, if NOX or other ROS-generating systems are also activated, NO bioavailability changes as a result of its scavenging by superoxide, and thus, the groups of signaling pathways regulated by NO are subsequently altered [144, 145].

Donors have also been used to show how NO affects T cell function. Treatment of T cells with low doses of NO produced by 10 μM DETANO led to increased cGMP and differentiation of Th1 cells in mice and humans [146]. These cells produce IFN-γ and appear to increase IL-12R selectively. In contrast, CD4$^+$ CD25$^-$ T$_{reg}$ cells treated with NO generated by 100 to 200 μM DETANO (200–400 nM steady-state NO for 24 h [147]) mediated a cGMP-independent but p53-dependent increase in proliferation and conversion of CD4$^+$ CD25$^-$ to CD4$^+$/CD25$^+$ T$_{reg}$ cells (called NO-T$_{reg}$ by the authors). These new NO-T$_{reg}$ cells represent an additional population of immunosuppressive T$_{reg}$ cells that do not express FKHD box P3 or TGF-β but instead, produce IL-10. The regulation of NO by this pathway is mediated by a p53-IL-2 pathway and acts to bypass IL-10-mediated conversion of CD4$^+$/CD25$^-$ to CD4$^+$/CD25$^+$ T$_{reg}$. This response is likely to occur in localized regions such as within lymphoid tissue, tumor microenvironment, or at sites of injury where NO production from immune and adjunct immune cells (particularly in humans) may occur. Importantly, the effect of high levels of NO in this setting serves to limit the M1/Th1 response.

Species-specific NO production

Much of the data that described the relationship between NO and immune signaling was derived from tumor cell lines or rodent macrophages, which are readily available sources of NO. However, in humans, although macrophages express *NOS2* mRNA and iNOS protein, the levels of expression and the immune in-

duction required to elicit expression are significantly different from rodents [148]. iNOS protein and NO production levels are remarkably lower, and induction is dependent on synergistic combinations of immune signals in human macrophagic cells. This species difference is most likely a function of the human iNOS promoter [149–152] and not the activity of iNOS itself [153]. To illustrate this point, **Fig. 5** provides a comparison map of the iNOS promoter region from the human and mouse *NOS2* genes. There is a significant mismatch between these promoters that is likely to account for the notable differences in the regulation of gene induction between mice and humans. In rodents and humans, *NOS2* mRNA expression is not restricted to macrophagic cells, and iNOS can be found in a number of cell types, including epithelial cells [154, 155], astrocytes [156], endothelial cells [157, 158], as well as other cell types [159]. Again, multiple differences in the induction of human *NOS2* compared with mouse *NOS2* are observed in these adjunct immune cells. Interestingly, human hepatocytes are known to express iNOS abundantly [20, 160–165] under normal conditions, and human neurons (not commonly associated with immune responses) ectopically express iNOS in chronic neurodegenerative diseases such as Alzheimer's disease [166].

Species and tissue-specific differences are also found for mechanisms that promote ROS production and consequently, may affect NO bioavailability and the NO/RNS-ROS balance. For example, rat macrophages express the NOX2-oxidative burst system and when stimulated with phorbol esters, rapidly produce abundant O_2^-. The rate of release, however, is approximately tenfold slower than the rate displayed by activated human neutrophils [167]. Part of the observed difference in O_2^- production is likely a result of differences in cellular location of the NOX proteins. Higher levels of ROS production by immune cells including microglia have also been noted in humans compared with rodents [168, 169].

The combined data on rodent versus human NO and O_2^- production strongly suggest that in general, ROS production is a predominant feature of activated human macrophages, neutrophils, and monocytes, and the equivalent murine immune cells generate a combination of O_2^- and NO and in some cases, favor NO production. As the balance in the levels and rates of production of NO and O_2^- dictates oxidative versus nitrosative stress, these differences may be crucial to understanding how immune responses are regulated in a species-specific manner. Overall, the wide varieties of adjunct-immune cells plus tissue-specific requirements for varying levels of immune protection provide plasticity to the immune response. This is particularly useful, as pathogen challenges change constantly.

The impact of NO signaling on an innate immune response—classical activation

Induction of the proinflammatory phase of an innate immune response is one of the initial, functional outcomes of the immune activation process and has been defined as "classical activation". Classically activated macrophages (also called M1 macrophages) have been well-described and are associated with the production and release of proinflammatory cytokines such as TNF-α, IL-6, and IL-1β, of proteases (e.g., MMP-9), and of NO/RNS and O_2^-/ROS. In addition to a role in pathogen eradication, the early increase in ROS, followed by increasing production of NO, results in a rapidly changing NO/RNS profile within the immune cell. As discussed above, the localized level of NO/RNS will dictate the type of reactive pathway profile and signaling during this phase of the response. However, as this process continues over time, secondary factors that influence the levels of NO and ROS, as also described above, will further modify the choice of pathways that is used. In the following sections, we will address some of the pathways that may participate in the integrated signaling, with emphasis on NO/RNS-mediated regulation of cross-talk between the proinflammatory and tissue-resolution pathways (**Figs. 6** and **7**).

NO and proinflammatory genes

Microarrays have been used to characterize the types of immune genes whose regulation is modified by NO. Using human macrophage cell lines treated with NO donors for varied periods of time, ranging from 3 to 24 h, Turpaev et al. [170–172] have identified specific categories of NO-sensitive genes. These include genes encoding transcription factors (e.g., *c-Fos*, *c-Jun*, *c-Maf*), regulators of cell cycle (e.g., cyclin A2, *p21/Cip1*), mitochondrial oxidative phosphorylation (ATPase 8 and *Nd4*), and many other cell pathway regulators, including *HO-1* and H-ferritin. Cytokine-related genes are also modified and are up-regulated as in the case of *TNF-α*, *IL-8*, and *MIP-1α* or down-regulated as *TRAF-1*-associated factor and *SLC7A6* (*LAT2*). In some cases, however, it was not NO but rather RNS that was the critical regulatory molecule. By using a NO trapping agent, 2-phenyl-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide, which generates NO_2 and ultimately N_2O_3, Turpaev et al. [172] showed that nitrosation chemistry is a primary driving force for expression of some proinflammatory genes. *TNF-α* expression is particularly increased under these conditions and to superactivated levels [172]. Activation of p38 MAPK pathways was also shown to be driven primarily by NO and RNS during classical activation. Gene regulation by nitrosative chemistry leads to additional levels of complexity. For example, a zinc thiolate ligand of the sheddase TACE (ADAM17) activates TNF-α in human monocytes, thereby increasing NF-κB and ultimately increasing expression of cytokines such as IL-8 and IL-6 [173]. Although the interconnection between IL-8 induction pathways may not be specific to nitrosation, it is interesting to speculate whether the expression of IL-8 can be used as a "surrogate" marker of nitrosative stress in diseases where high levels of NO can be generated.

For many of the immune pathways that are regulated by NO, ROS, PGs, and the other species represented in Fig. 1, NF-κB is critical in orchestrating the innate immune response outcomes [174]. In unstimulated cells, NF-κB subunits are sequestered into the cytoplasm by inhibitory protein complexes. Multiple stimuli (e.g., LPS or TNF-α) initiate a phosphorylation cascade that causes the inhibitory proteins to be degraded proteasomally, allowing NF-κB to enter the nucleus where its modulation of gene induction dictates, in part, the pattern of immune activity. NF-κB activity is also regulated by redox-related mechanisms, particularly as NF-κB activation is biochemically regulated by intracellular thiol status [175]. ROS and RNS species have been shown to alter NF-κB signaling. For example, a short burst of NO augments the activation of NF-κB in LPS or TNF-α-stimulated cells [176, 177], and other studies show that longer duration of NO exposure led to inhibition and served as a negative-feedback signal [178–180]. Other observations with ROS show that low levels of H_2O_2 can similarly enhance NF-κB activity [181, 182], and higher H_2O_2 (>100 μM) inhibited its activity [178–180]. These redox-mediated inhibitory reactions involve critical cysteine thiolates necessary for DNA binding [183–185]. Also, oxidation of methionine-45 prevents IkBα degradation and subsequent nuclear translocation of NF-κB [186]. Excessive oxidative stress by RNS or ROS has been shown to inhibit NF-κB activity by modulating the critical cysteine thiols of NF-κB subunits [187]. Additionally, NF-κB is regulated partially by redox-sensitive phosphatases and kinases, suggesting that redox regulation of NF-κB may be multifactorial [188]. The overall effect of NO and ROS on NF-κB activity follows a biphasic pattern, where low NO/ROS levels increase NF-κB activity, but higher NO/ROS levels inhibit NF-κB-mediated gene transcription [189]. It should be noted that many of these observations are cell- and mechanism-dependent.

NO and regulation of anti-inflammatory pathways

Proinflammatory, classical activation is followed rapidly in time by an anti-inflammatory repair phase that ideally leads to wound-healing and tissue restoration, which is ultimately the desired outcome of a successful innate immune response. These time-delayed phases of the innate immune response are initiated by the original pathogens or injury but are rapidly re-enforced by the actions of anti-inflammatory cytokines released from macrophages and microglia that act in an autocrine manner. Anti-inflammatory cytokines also arise from accessory immune cells such as astrocytes or from Th2 T_{reg} [190, 191]. The four major anti-inflammatory cytokines involved in tissue repair and wound-healing are IL-4, IL-13, IL-10, and TGF-β [191–193]. These cytokines initiate down-regulation of the proinflammatory phase and induction of tissue reconstruction and repair. NO/RNS and ROS play critical roles in regulating these pathways. Much like a traffic cop, the level of NO/RNS redirects signaling by reducing one pathway and enhancing another (Fig. 6).

An interesting example of this flexible, level-dependent regulation is found in the interaction between NO and eicosanoids. Macrophage activation by LPS or other immune stimuli leads to increased iNOS activity and NO, which suppresses COX2 enzymatic activity [194] and supports the long-term expression of *COX2* mRNA [195]. However, as the activity of iNOS decreases, there is a marked increase in PGE2 by COX2 stimulation that leads to increased cAMP-mediated processes. As NO is a direct activator of soluble guanylyl cyclase and increased cGMP and as cGMP- and cAMP-dependent pathways often regulate cell processes in an antagonistic manner, the increased cAMP-based process may reflect initiation of competing pathways. This type of competition is observed in LPS-activated monocytes, where cGMP inhibits IL-1β expression, and PGE2/cAMP inhibits TNF-α [196, 197] (Fig. 6). Other studies examining phosphodiesterase inhibitors have shown that cAMP and cGMP play a key role in regulating IL-1β and TNF-α [198]. This implies that under conditions of high NO output, increased TNF-α is favored. However, when NO levels decline (such as scavenging by ROS), then COX2/PGE2 and cAMP levels increase, which may favor IL-1β production and activity. Another important aspect is that PGE2/cAMP mediates increased levels of AG, IL-10, and IL-1R antagonist and suppresses IL-12, whereas cGMP does not [199, 200]. These observations suggest that with respect to NO and ROS balance, modulation of cGMP and cAMP plays a role in polarization of the immune system.

During transition from proinflammatory to resolution phase, numerous pathways are used to down-regulate the initial killing/proinflammatory signaling mechanisms to homeostatic levels. These feedback signals include switching from cGMP- to cAMP-mediated pathways within the macrophage, as we described above, and others include the induction of decoy adaptor proteins [201] or selective "suppressor" proteins, such as suppressor of cytokine signaling [202]. Additionally, surface cytokine receptors modulate autocrine feedback loops, which when activated, impact NO/RNS- or ROS-mediated regulation on several levels. For example, production of O_2^- is linked to TNF-αR1 signaling on the macrophage plasma membrane. Kronke and colleagues [203] have recently shown that TNF-αR1 is directly coupled to a NOX by the binding of riboflavin kinase to the receptor and the p22phox subunit in the membrane. This kinase is specifically required for TNF-α-mediated ROS production but is not a component of LPS-stimulated ROS. As mentioned earlier, tethering the oxidase to specific locations helps to focus the actions of the ROS that are produced.

When high levels of NO are produced simultaneously, not only can the NO act as a local antioxidant to protect membranes against ROS, but NO can also react with the superoxide produced by TNF-αR1 activation to generate nitrite, thus reducing NO levels. This mechanism may be an important protection against TNF-α-mediated cell death and clearly demonstrates the functional significance of the NO/ROS balance.

NO/RNS-mediated induction of HO-1 or of H-ferritin is an additional cellular mechanism that will further limit TNF-α-induced ROS within cells [204]. HO-1 is a microsomal enzyme that cleaves heme-containing proteins to produce the antioxidants, biliverdin and CO [205]. Part of the enzymatic action, however, is to increase cytosolic-free iron levels and consequently, H-ferritin levels [206]. H-ferritin further contributes to the antioxidant protection of the cell by mediating activation and translocation to the nucleus of the transcription factor, NRF2 [207]. Once in the nucleus, NRF2 binds to regulatory promoter elements including the ARE, which is responsible for induction of antioxidant protection mechanisms [208–210]. HO-1 further shifts the macrophage-immune function to the repair phase by increasing anti-inflammatory cytokine production, which in turn, increases HO-1 [211]. The synergistic action of HO-1 helps to re-enforce the suppression of proinflammatory factors such as iNOS, MPO, and COX by degrading these heme-containing enzymes. The lack of heme further prevents the re-insertion of heme into these critical immune enzymes, thereby blocking their function [212]. Low levels of iNOS, COX2, and MPO are associated with immune-resolution phases. In addition, CO reduces the function of TLR p38 MAPK signaling [130, 213]. Finally, H-ferritin has been shown to prevent JNK activation and TNF-α-mediated cell death by sequestration of iron [214]. Thus, HO-1 and CO-mediated pathways can play an important part in the protection of tissue as well as ameliorating inflammation.

The shift from NO/RNS to ROS-mediated regulation during the transition from classical activation to alternative activation involves other unique mechanisms and provides additional opportunities to understand the dual nature of these responses. Many immune receptors, including those that are activated by anti-inflammatory cytokines such as IL-4 are associated with JAK. This association requires JAK-mediated transphosphorylation for activation of the signaling pathways. Upon binding to the receptor, multiple tyrosine residues are phosphorylated and in turn, activate STAT6 or other downstream pathways. Ultimately, genes involved in tissue repair and restoration such as *Ym-1* (also known as chitinase 3-like 1) or *AG1* are induced, and their corresponding proteins are expressed. Part of the JAK-mediated activation process involves production of O_2^- by NOX5 and/or NOX1 via an insulin receptor substrate/PI3K pathway [215]. One of the targets of the ROS generated in this activation process is PTP1B. This phosphatase dephosphorylates the IL-4R and hence, is responsible for its inactivation. Other PTPs such as SHP-1 or CD45 are also critical to limiting signaling initiated by JAK-based activation [216] and are part of a general immune signaling mechanism. Interestingly, ROS play a critical role in prolonging the action of IL-4. PTPs contain cysteinyl residues in their catalytic site, which when oxidized by ROS, results in inactivation of the phosphatase [217, 218]. Consequently, kinase activity is extended, and IL-4 signaling is enhanced. The overall result is maintained suppression of proinflammatory genes and up-regulation of alternative activation genes, which favor repair and restoration of the tissue. RNS have opposing roles when compared with ROS. Nitrosation of PTPs prevents irreversible oxidation, thus allowing the phosphatase to continue to function in a normal manner [219, 220]. Interestingly, *Leishmania* survival within macrophages is dependent on manipulation of PTP activity; in this case, the PTP is SHP-1. Blanchette and coworkers [221] propose that continuous activation of SHP-1 leads to down-regulation of iNOS induction and decreased NO production, resulting in reduced toxicity to the pathogen. A similar effect is observed in virally infected glia cells, where NO levels are reduced by altering SHP-1 [222].

NO impact on adaptive immunity—immunosuppression and tissue-restoration response

NO is also involved in immunosuppression by regulating circulating immune cells. For example, MDSCs can be activated by NO-mediated increases in cGMP, which in turn, facilitates their binding to CTLs and reduces T cell proliferation [223]. When cell-to-cell contacts are formed, expression of AG and iNOS is required to induce apoptosis [224]. Increased iNOS activity is also found in mature DCs, where NO is associated with suppression of T cell proliferation. Furthermore, when activated by IFN-γ, TNF-α, or IL-1α/β, MSCs produce chemokines and iNOS, which leads to the immunosuppression of those T cells in the vicinity of the MSCs [225]. The resulting increase in chemokines and iNOS leads to the attenuation of T cell responsiveness. In general, T cell responses are decreased by NO. As shown in Fig. 7, the immune regulation in this case includes T cell populations with distinct immunosuppressive mechanisms ($CD4^+/CD25^+$) through the induction of HO-1 and subsequently, CO through IL-10 production and through increased activation of latent-to-active TGF-β via nitrosation [223, 226]. In this manner, NO can be viewed as switching Th1 to Th2 responses. In humans, where NOS2 activity and NO levels are low, a different story has emerged. Rather than NO as the primary factor, the tryptophan-degrading enzyme, IDO, plays the primary immunosuppressive role. Ren et al. [227] have shown that MSCs express high levels of IDO when stimulated with IFN-γ and that inhibitors of IDO blocked the MSC-mediated reduction in T cell activity. Similar IDO-mediated immunosuppression has been found for other human immune cells [228–230].

It is interesting to note that AG also plays a role in T cell regulation. An elegant study by Pesce et al. [231] recently demonstrated a NO-independent role for AG1 in alternatively activated macrophages in mice chronically infected with the parasite trematode, *Schistosoma mansoni*. Although AG expression in macrophages is prominently associated with Th2 responses in infected tissue and acts to promote repair processes, including fibrosis, Pesce and colleagues [231] demonstrated that part of its function is to sequester arginine away from $CD4^+$ effector T cells within the same tissue. By competing for arginine, AG activity suppressed T cell proliferation by depleting the available levels of arginine, In this case, however, the local effector T cell population was associated with IL-4 and IL-13 production and Th2-mediated events. Thus, suppression resulted in a reduced Th2 response. When arginine was added back, the inhibition of T cell proliferation was removed, and the "brakes" on an overactive Th2 response were reapplied. Interestingly, genetic deletion of AG1 resulted in decreased survival as a result of increased liver fibrosis and portal hypertension in *Schistosoma*-infected liver, not proinflammatory cytokine activity, and shows unequivocally that Th2 responses can be toxic.

Overall, low NO may favor Th2, and high NO may augment Th1 responses and suggests that a balance between NO and ROS may help to determine immune polarity. During the immune repair/restoration phase, the collective activity of IL-4, IL-13, IL-10, and TGF-β helps to suppress iNOS expression and NO, shifting in favor of ROS [232, 233]. In humans, the relatively higher production of ROS and the lower production of NO, although not absolutely required for IDO action [230], clearly play a role in promoting IDO-mediated immunosuppression.

NO and revascularization

Besides regulating immune signaling pathways involved in M1 and M2 states, NO/RNS and ROS participate in tissue restoration mediated by macrophages by helping to orchestrate the clean-up of debris, the restructuring of ECM and cell proliferation, and the formation of new blood vessels [191, 234]. For example, macrophage NO production contributes to angiogenesis by increasing VEGF, a key growth factor for new

blood vessels. This is accomplished through two principle immune pathways: induction and stabilization of the transcription factor, HIF-1α, and formation of PGE2. HIF-1α is regulated by NO (and in some tissues, by PGE2), cytokines including IL-1β and TNF-α, as well as growth factors. HIF-1α serves as a cellular hypoxia sensor and acts to induce genes that promote vasodilation, vascular permeability, glucose uptake, and angiogenesis [235, 235–237]. Each of these features is required to revascularize an injured tissue during immune repair. Modulation of NO levels is critical during regulation of these tissue-restoration activities. For example, binding of VEGF to endothelial cells induces eNOS serine-1179 phosphorylation, culminating in cellular proliferation [238]. MMPs, such as MMP-9, are also activated by NO (at a 50- to 500-nM range), where the increased proteolytic activity is required for restructuring the surrounding matrix for neovascularization [239]. This level of NO is associated with proliferation and antiapoptosis though activation of Akt and ERK and appears to be critical in the tissue-restoration phase [28].

Acute versus chronic inflammatory disease

NO and redox molecules are critical factors during the progression of an immune response from inflammation and immunosuppression to tissue-restoration phases in vivo (**Fig. 8**). Importantly, redox molecules mediate different inflammatory outcomes, depending on the specific type (e.g., chronic or acute). Thus, the effects of NO/RNS and ROS are context-dependent. This concept is particularly relevant in regard to the NO generated from iNOS and eNOS isoforms. Here, we will briefly compare and contrast some specific roles of these NOS isoforms during acute and chronic inflammation to illustrate the temporal and concentration dependence of NO with respect to outcome.

During acute inflammation, eNOS and iNOS mediate different effects depending on the type of injury. In experimental arthritis models involving streptococcal cell wall injections, eNOS was found to be deleterious, and iNOS activity was protective [240–242] and suggests that striking differences in the actions of these NOS isoforms can be found under specific conditions. Similarly, Chen et al. [243] demonstrated iNOS-mediated protection against hepatic apoptotic cell death seen in models of sepsis and hepatitis. Both models show bacterial product activation of TLR, which leads to macrophage and hepatocyte activation. However, as shown in eNOS knockout mice, the cytotoxic action of eNOS most likely results from its ability to up-regulate iNOS and other proinflammatory factors via NF-κB [244]. In contrast, elevated iNOS expression mediated by NPR-A (a.k.a., NPR1) caused vasodilation and vascular dysfunction, which are associated with a variety of complications in a model of endotoxic shock [245].

Similarly, contrasting effects of NOS isoforms occur during IR injury, where eNOS is protective, but iNOS is detrimental. In most IR models, eNOS has a positive effect, and iNOS is associated with toxicity. In part, this is because reperfusion injury focuses on the endothelium. In this context, low NO flux generated by eNOS prevents leukocyte recruitment and associated tissue damage through scavenging of ROS in the early phase [246]. However, when iNOS expression increases, NO levels rise and induce tissue injury, especially to the endothelial cells. NO potentiates the hepatic oxidative injury in warm IR [243]. In IR injury, preservation of the endothelium is critical for damage control. Thus, the beneficial effects of specific NOS isoforms depend on the type of primary damaging event. During sepsis or infection, the key initial events involve monocyte infiltration and concomitant elevated levels of NO. In contrast, IR injury occurs at the endothelium, where low flux NO protects against ROS-mediated endothelial damage. Elevated levels of NO generated from iNOS are deleterious when expressed in or near vascular beds. A comparison of these acute models of inflammation suggests that the location and temporal factors determine the outcome of

eNOS and iNOS expression. When associated with immune cells, eNOS is detrimental, and iNOS is beneficial. However, when eNOS and iNOS are associated with vascular beds, the reverse is true, and eNOS mediates a positive outcome, and iNOS is detrimental. It should be noted that iNOS transfection does prevent hyperplasia of the vascular smooth muscle cell in restenosis [247, 248]. Thus, careful evaluation of the context dependence of NOS isoforms should be considered when assessing outcome.

Similar NO dichotomies occur in other inflammatory diseases. For example, in cancer, iNOS has positive and negative effects on prognosis [249]. As discussed in Introduction, it was shown that NO is involved in antitumorigenic processes, especially with respect to leukemias [250]. Mouse models show that iNOS and p53 knockout mice are prone to increased leukemias, primarily because of the role of NO during p53-mediated apoptosis. Studies suggest that iNOS expression in the liver can mediate apoptosis of circulating cancer cells, thus reducing metastatic burden. However, many cancers have p53 mutations. In this context, NO potentiates the oncogenic behavior of mutated p53. Many solid tumors have shown that iNOS within the tumor leads to poor patient prognosis [249]. In animal models, iNOS correlated with increased aggressiveness of colon cancers [251]. As iNOS produces different levels of NO, specific levels of NO generated by iNOS can activate HIF-1α, Akt, and ERK pathways, which then lead to increased proliferation, anti-apoptosis, and metastatic mechanisms such as epithelial mesenchymal transition [249]. This would lead to increased proliferation, dissemination, and a higher-grade tumor. In addition, NO would lead to depression of lymphocytes with increased maturation of MDSCs and DCs as well as conversion of T_{reg} to $CD4^+/CD25^+$ [251]. Taken together, these observations suggest that immunosuppression, consistent with the immune state that is present during wound-healing, serves to promote a more aggressive tumor phenotype.

CONCLUSIONS

The multifaceted role(s) of redox biology demonstrate complex and yet defined functions for reactive species in the immune response (Fig. 9). As immunotoxins, the varieties of RNS and ROS intermediates serve as arsenals that fight different pathogens. In addition, the development of resistance to these reactive intermediates suggests a continuous evolution of pathogen and host responses. The difference between murine models and humans with respect to regulation of these molecules indicates that adaptation to different biological stressors may have provided increased versatility in the overall scheme to fight pathogens and tumors. Regulation of immunity suggests that concentration, timing, and location of redox regulators determine outcome. The participation of NO and redox-related molecules during inflammation and tissue-restoration processes indicates the importance of redox-active molecules in coordinating these events. The identification of context-dependent outcome, with respect to redox intermediates, suggests that "the devil is in the details", and within such details are new ways to look at disease and new therapeutic opportunities.

Footnotes

ADAM17 a disintegrin and metalloproteinase domain 17
ADMA asymmetrical dimethylarginine
AG arginase
Akt-/ERK-P Akt-/ERK-phosphorylation
ARE antioxidant response element

BH$_4$ tetrahydrobiopterin

CBS   cystathione β-synthase

CO   carbon monoxide

CO$_2$ carbon dioxide

DETANO   diethylenetriamine-NO

DUOX 1/2   dual oxidase 1/2

eNOS   endothelial NOS

FA   fatty acids

FKHD   forkhead

H$_2$O$_2$ hydrogen peroxide

H$_2$S   hydrogen sulfide

HCys   homocysteine

HIF-1α   hypoxia-inducible factor 1-α

IR   ischemia reperfusion

LPG   lipophosphoglycan

MDSC   myeloid-derived suppressor cells

MMP-9   matrix metalloprotease 9

MSC   mesenchymal stem cell

N$_2$O$_3$ nitrogen trioxide

NO$_2$ nitrogen dioxide

NO$_2^-$ nitrite

NOHA   N-hydroxyarginine

nNOS   neuronal NOS

NOX1/2/3/4/5   NADPH oxidase 1/2/3/4/5

NPR   natriuretic peptide receptor

NRF2   nuclear factor (erythroid-derived 2)-like 2

O$_2^-$ superoxide anion

ONOO$^-$ peroxynitrite

PPARγ   peroxisome proliferator-activated receptor γ

PTP   protein tyrosine phosphatase

RNS   reactive NOS

SAG   sodium antimony gluconate

SAM   s-adenosyl-methionine

SHP-1   Src homology 2-containing protein tyrosine phosphatase, nonreceptor type 6

T$_{reg}$ T regulatory cell

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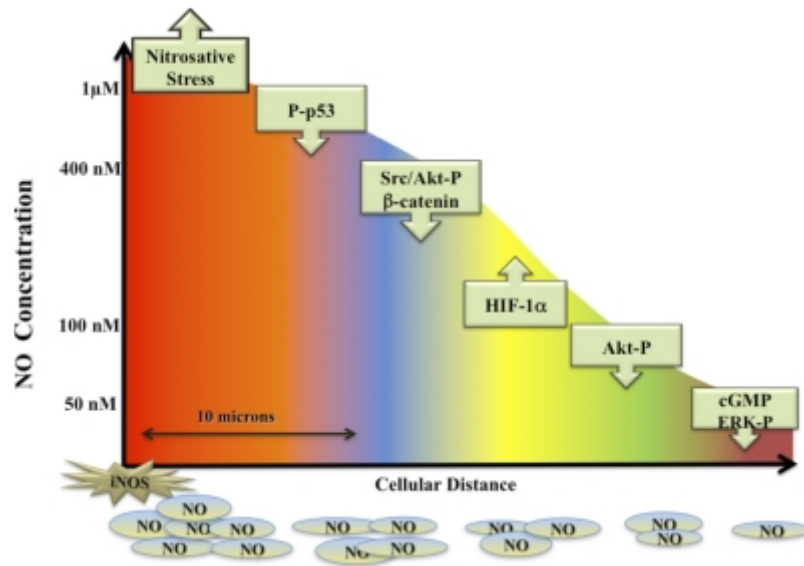Feedback

Figures and Tables

Figure 1.
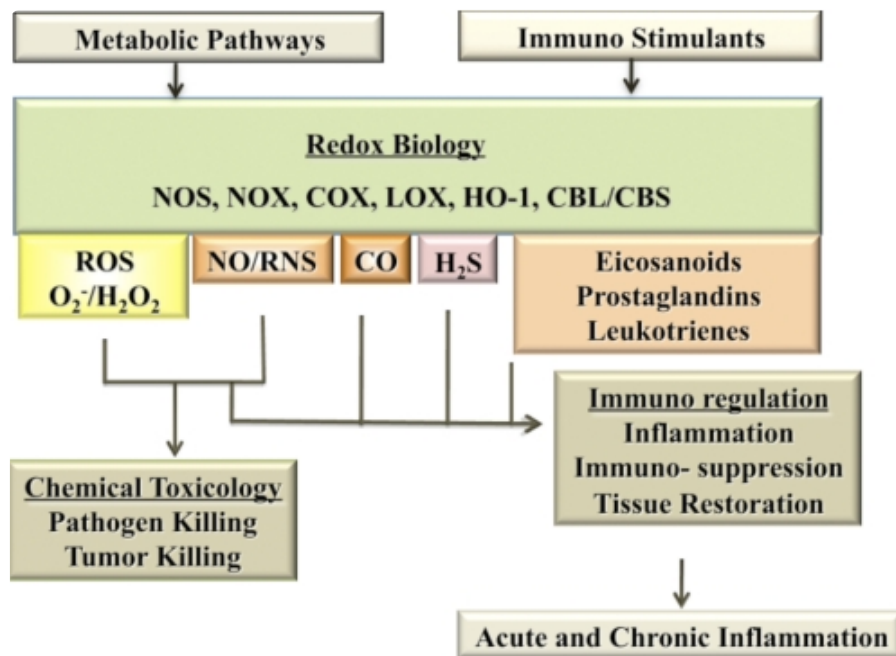
The immunotoxic and immunoregulatory aspects of redox-based biology.

The redox species and sources are: NO and RNS derived from NOS; ROS derived from NOX; arachidonic acid products derived from COX and lipoxygenase (LOX); CO derived from HO-1; and H_2S from CBS and cystathionine β-lyase (CBL). Of the multiple redox species involved in immunity, NO/RNS and ROS are critical to defense against pathogens and for regulation of the response.

Figure 2.

The NO chemistry of the phagosome.

This diagram depicts the different nitrogen oxide and ROS chemistry that can occur within the phagosome to fight pathogens. The presence of NOX2 in the phagosomes serves two purposes: one is to focus the nitrite accumulation through scavenging mechanisms, and the second provides peroxide as a source of ROS or FA generation. The nitrite (NO_2^-) formed in the acidic environment provides nitrosative stress with $NO/NO_2/N_2O_3$. The combined acidic nature and the ability to form multiple RNS and ROS within the acidic environment of the phagosome provide the immune response with multiple chemical options with which it can combat bacteria.

Figure 3.

Diagrammatic view of critical interactive connections between NO and ROS-mediated metabolic pathways.

DHFR, Dihydrofolate reductase; SAH, S-adenosyl-HCys; ODC, ornithine decarboxylase.

Figure 4.

The effect of steady-state flux of NO on signal transduction mechanisms.

This diagram represents the level of sustained NO that is required to activate specific pathways in tumor cells. Similar effects have been seen on endothelial cells. These data were generated by treating tumor or endothelial cells with the NO donor DETANO (NOC-18) for 24 h and then measuring the appropriate outcome measures (for example, p53 activation). Various concentrations of DETANO that correspond to cellular levels of NO are: 40–60 μM DETANO = 50 nM NO; 80–120 μM DETANO = 100 nM NO; 500 μM DE-TANO = 400 nM NO; and 1 mM DETANO = 1 μM NO. The diagram represents the effect of diffusion of NO with distance from the point source (an activated murine macrophage producing iNOS) in vitro (Petri dish) generating 1 μM NO or more. Thus, reactants or cells located at a specific distance from the point source (i.e., iNOS, represented by star) would be exposed to a level of NO that governs a specific subset of physiological or pathophysiological reactions. The x-axis represents the different zone of NO-mediated events that is experienced at a specific distance from a source iNOS producing >1 μM. Note: Akt activation is regulated by NO at two different sites and by two different concentration levels of NO.
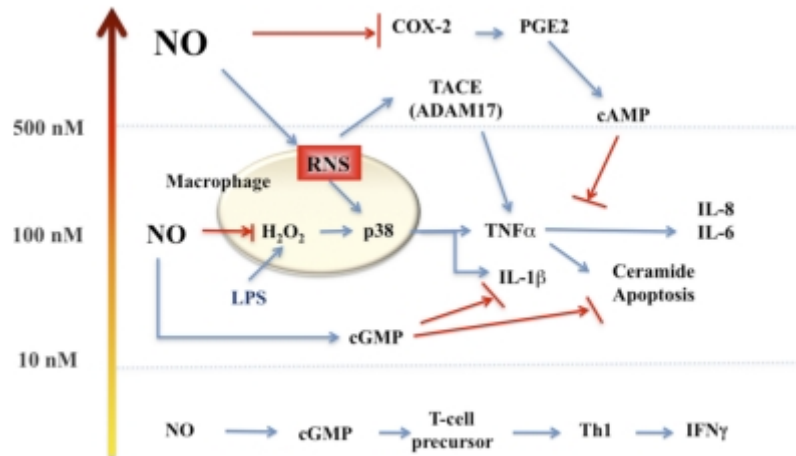
Figure 5.
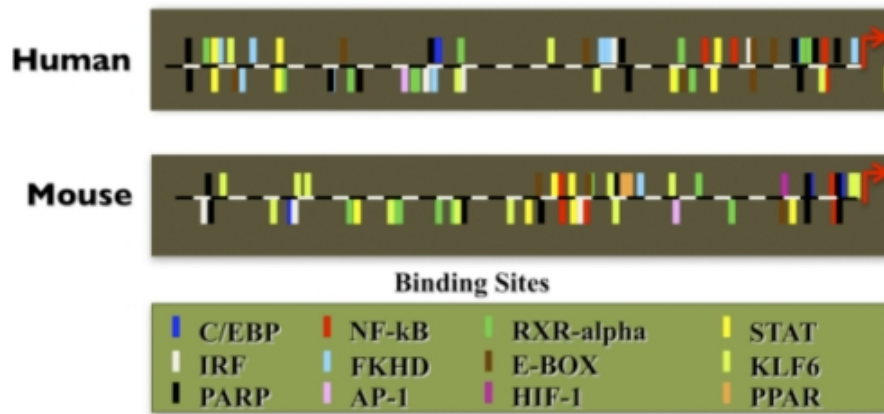
Comparison of the promoter region of *NOS2* between human and murine.

Promoters′ information was obtained from the Genomatix Software Suite. Each bar represents a particular transcription factor binding site. Upward bars represent the bind site located on the sense strand of the DNA, and downward bars represent the binding sites located on the nonsense strand. IRF, IFN regulatory factor; PARP, poly(ADP-ribose) polymerase; RXR-α, retinoid X receptor α; E-BOX, E-box binding factors, contains binding domains bHLH-ZIP and bZIP; KLF6, Kruppel-like factor 6.
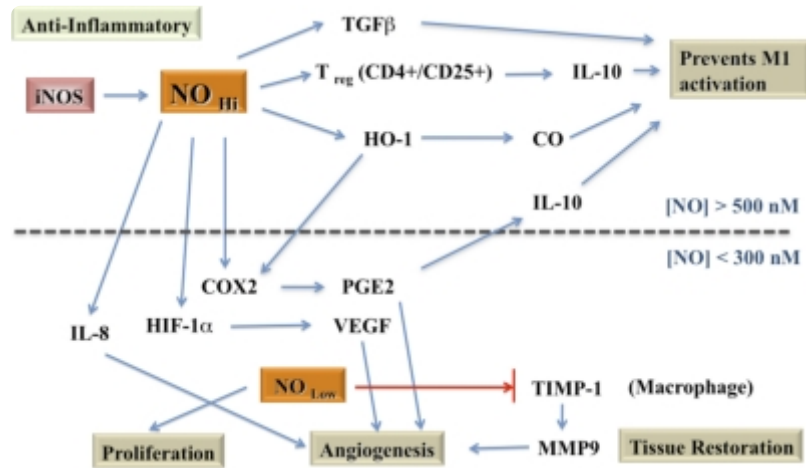
Figure 6.

Mechanisms of NO that activate a Th1 phenotypic response.

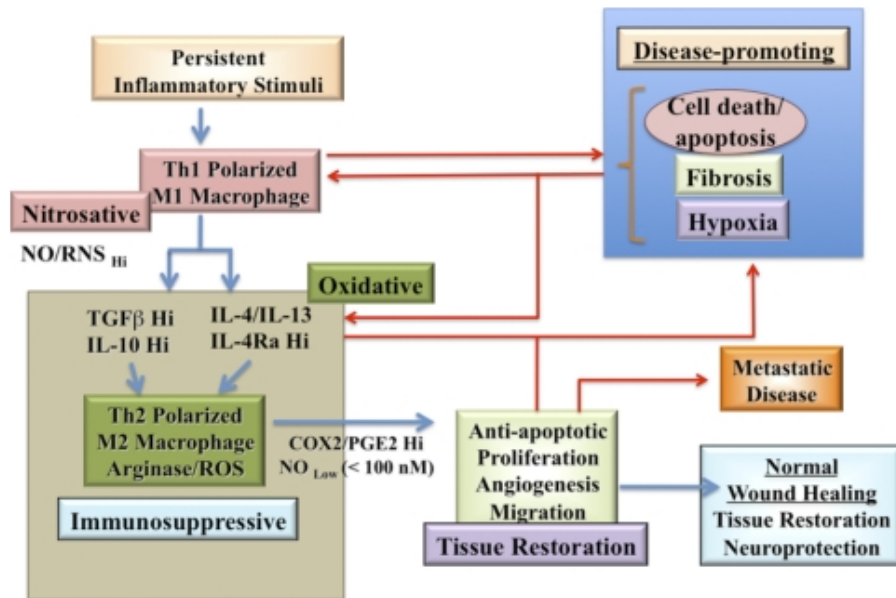For description of regulated pathways, please see text.

Figure 7.

Mechanisms of NO in immunosuppression and tissue restoration.

For description of regulated pathways, please see text.

Figure 8.

A flow diagram of NO in inflammation and tissue-restoration response.

Blue lines represent the linear progression from initial insult to tissue restoration. A fatal loop is found in diseases such as cancer, where the processes are restimulated, and leads to chronic conditions or a "wound that never heals". The lines in red show pathways that contribute to perpetuation of chronic disease and failure to resolve tissue-restoration processes correctly.

Figure 9.
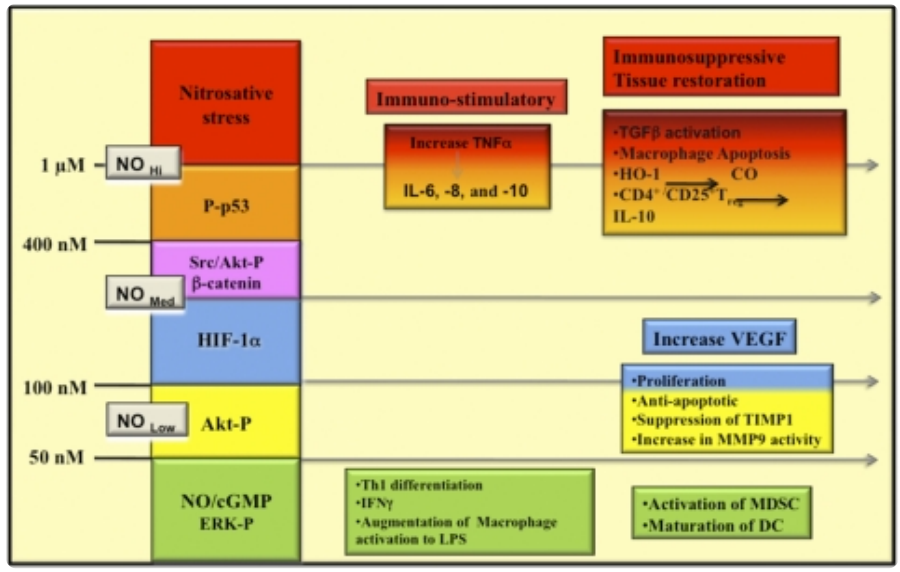
A summary of levels of NO required to elicit different mechanisms of the inflammation and tissue-restoration response.