Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity

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Nitric oxide, nitric oxide derivatives and reactive oxygen intermediates are toxic molecules of the immune system which contribute to the control of microbial pathogens and tumors. There is recent evidence for additional functions of these oxygen metabolites in innate and adaptive immunity; these functions include the modulation of the cytokine response of lymphocytes and the regulation of immune cell apoptosis, as well as immunodeviating effects. Components of several signal transduction pathways have been identified as intracellular targets for reactive nitrogen and oxygen intermediates.

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Abbreviations

IFN	interferon
LPS	lipopolysaccharide
MPO	myeloperoxidase
NK	natural killer
NO	nitric oxide
NOS	NO synthase
RNI	reactive nitrogen intermediate
ROI	reactive oxygen intermediate
SOD	superoxide dismutase
TGF-β	transforming growth factor β
TNF-α	tumour necrosis factor α

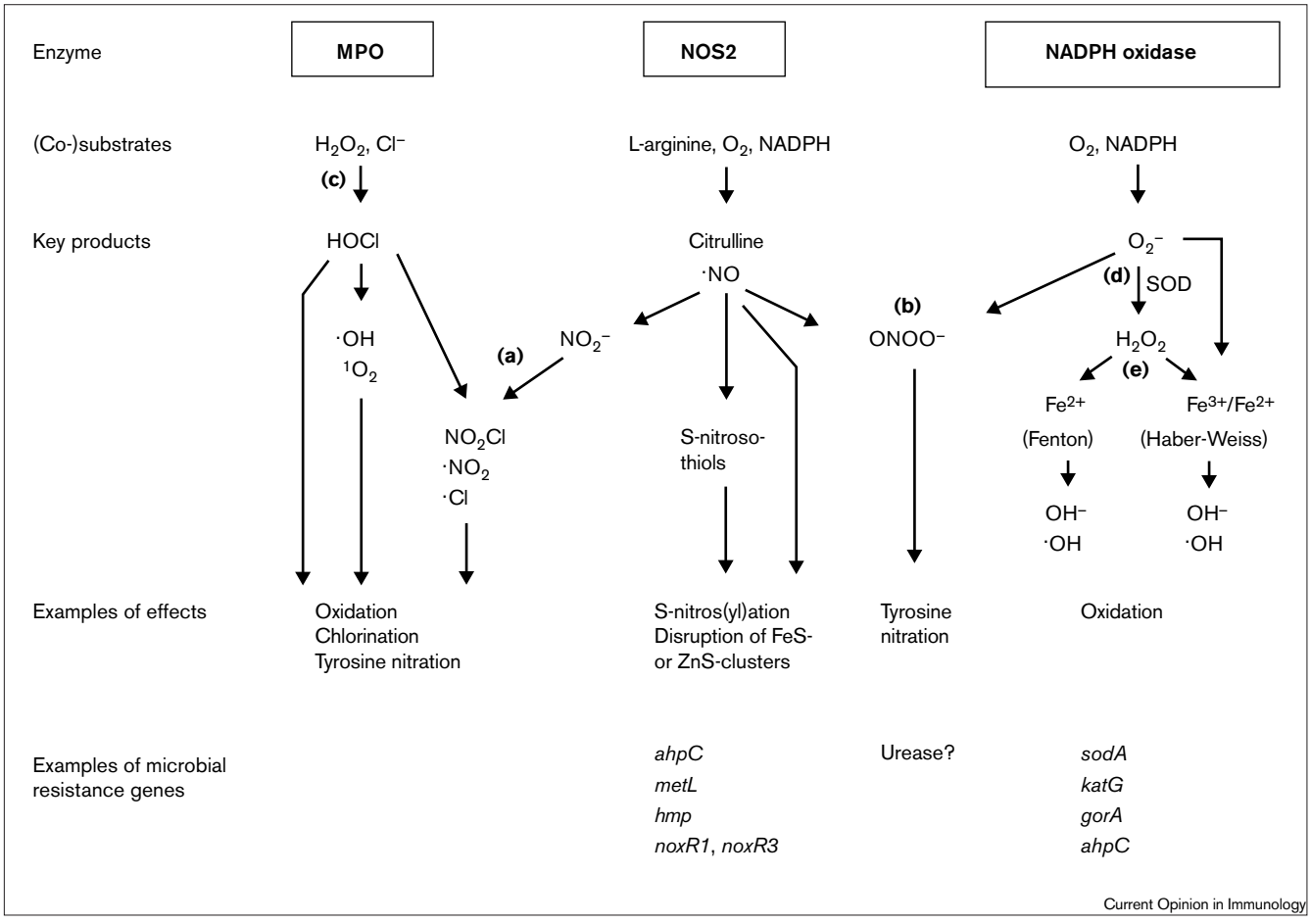
Introduction

The main function of the immune system is the defense of the host organism against infectious agents and malignant tumors. Macrophages, neutrophils and other phagocytic cells are key components of the antimicrobial and tumoricidal immune responses, which is due to the fact that these cells are capable of generating large amounts of highly toxic molecules — reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (RNIs) (Figure 1).

The production of ROIs is initiated by NADPH oxidase, which becomes activated upon translocation of several cytosolic proteins (i.e. $gp40^{phox}$, $gp47^{phox}$, $gp67^{phox}$ and the Rho-family GTPase, Rac2) to the membrane-bound complex carrying cytochrome C ($gp91^{phox}$–$gp22^{phox}$–Rap1a). The activation of NADPH oxidase can be elicited by microbial products (e.g. lipopolysaccharide [LPS; also known as endotoxin] and lipoproteins), by IFN-γ, by IL-8 or by IgG-binding to Fc-receptors. The primary product of the reaction catalyzed by the NADPH oxidase is superoxide (O_2^-), which can be converted to H_2O_2 by superoxide dismutase (SOD), to hydroxyl radicals (·OH) and hydroxyl anions (OH^-) by the iron-catalyzed Haber-Weiss reaction or, after dismutation to H_2O_2, to hypochlorous acid (HOCl) and chloramines by myeloperoxidase (MPO) (reviewed in [1]; Figure 1). Although each of these oxygen metabolites exerts antimicrobial activity against extracellular bacteria, it is still not clear to what extent phagocytes other than neutrophils (notably macrophages) utilize ROIs for the control of intracellular microbes *in vivo*. It is important to bear in mind that an ROI (O_2^-) is also generated by the mitochondrial electron transport chain [2] and, under conditions of L-arginine depletion, by nitric oxide (NO) synthases (NOSs) (see [3] and references therein). Therefore, the production of ROIs is by no means restricted to phagocytic effector cells and there are more functions of ROIs in addition to antimicrobial activities.

All NOSs convert the amino acid L-arginine and molecular oxygen to L-citrulline and NO (·NO radicals or subsequent intermediates), and require NADPH, FAD, FMN, tetrathydrobiopterin (BH_4) and a thiol donor as cosubstrates and cofactors. Currently, there are three major NOS isoforms known that are named after the prototypic cell type from which the respective isoform was first isolated as well as after the main characteristic of their regulation. The neuronal NOS (also known as ncNOS or NOS1) and the endothelial NOS (ecNOS or NOS3) are constitutively expressed; they exist as preformed monomers in the cell, which dimerize and gain activity upon Ca^{2+} influx and binding of calmodulin. The macrophage NOS (macNOS, iNOS or NOS2), in contrast, is an inducible isoform that is absent in strictly resting cells, is strongly induced by cytokines and other immunological stimuli and is regulated on transcriptional and post-transcriptional levels involving a number of signal transduction pathways and molecules: Jak1/Stat1α/IRF-1; IκB/NF-κB; mitogen-activated protein kinases; protein kinase C; phosphatidylinositol-3 kinase; protein tyrosine phosphatases; and protein phosphatases 1 and 2A (reviewed in [4,5•]). The various NOS isoforms are also subject to regulation by the availability of L-arginine and BH_4, which are required for the formation of active NOS dimers. Virtually every cell of the immune system has been described to express one or several isoforms of NOS and most probably every type of mammalian cell is capable of generating NO. NOS activity (NOS1, NOS2) is found in the cytosol (attached to cytoskeletal proteins) but can also be targeted to plasmalemmal caveolae (NOS3) or localized in vesicles (NOS2) or in mitochondria (reviewed in [6,7]). The primary NO species that is synthesized by the NOSs has been a matter of debate because it is very much dependent on the

Figure 1



Pathways for the generation of ROIs and RNIs in antimicrobial effector cells. Phagocytes generate ROIs and RNIs by MPO, NOS2 or NADPH oxidase. There are several known interactions between these antimicrobial effector pathways: **(a)** the utilization of NOS2-derived nitrite (NO_2^-) in the MPO pathway, leading to the production of nitryl chloride (NO_2Cl) and nitrogen dioxide (NO_2); **(b)** the generation of peroxynitrite ($ONOO^-$) from nitric oxide (·NO) and superoxide (O_2^-); and **(c)** the consumption in the MPO pathway of peroxide (H_20_2) that **(d)** is generated from O_2^- by the action of SOD. **(e)** Ferrous iron (Fe^{2+}) and H_2O_2 will yield OH^-, ·OH and ferric iron (Fe^{3+}) (Fenton reaction). In the presence of O_2^-, Fe^{3+} will be reduced to Fe^{2+}, which then again allows for the conversion of H_2O_2 to ·OH (Haber-Weiss reaction). Microbes are equipped with a variety of defense mechanisms (resistance genes) that help them to survive the oxidative and nitrosative stress (see text). The expression of NOS2 is not restricted to phagocytes but is also found in other cells (e.g. fibroblasts, endothelial and epithelial cells, keratinocytes, and certain B and T cell lines). Some of the biological effects of NOS2-derived NO or of NADPH-oxidase-derived ROIs are likely to be also achieved by NO derived from constitutive NO synthases (NOS1, NOS3) or by ROIs generated in the mitochondrial respiratory chain (see text for examples).

composition of the intra- and extra-cellular micromilieu. In the strict absence of O_2^-, the ·NO radical will be formed whereas in the presence of ROIs, peroxynitrite ($ONOO^-$) and other RNIs might prevail (see [8] and references therein). Reaction of ·NO with SH-groups of free amino acids, of peptides or of proteins will give rise to S-nitrosothiols; this is not only a mechanism for scavenging NO but also serves to transport NO and is the molecular basis for many of the regulatory functions of NO described below (see [9] and references therein; see also Figure 1).

The original concept that the small quantities of NO generated in a pulsatile fashion by constitutive NOSs mainly fulfil regulatory functions required for normal homeostatic function of the vasculature and the central nervous system, whereas the high amounts of NO produced by NOS2 primarily exert antimicrobial and cytotoxic effects in the immune system, has recently seen several important modifications. In this brief update covering the advances of the past year, we will highlight new aspects of the interaction between infectious agents and ROIs/RNIs, outline some immunoregulatory activities of ROIs and NOS2-derived NO and discuss the evidence for a role of ncNOS/ecNOS-derived NO in the immune system.

Regulation of NO production

Cytokines

The most characteristic feature of NOS2 is its prominent regulation by activating cytokines (e.g. IFN-γ and TNF-α)

Table 1

The course of infections in transgenic mice deficient for oxygen-dependent antimicrobial effector mechanisms.

Defective molecule	Infectious agent	Route of infection; form of disease	Effect on disease severity	References
NOS2	Coxsackie virus B3	i.p.; myocarditis	↑	[112]*
	Ectromelia virus	i.v.; mouse pox (liver, spleen, lung)	↑	[113]†
	Herpes simplex virus type 1	s.c.; cutaneous infection, dorsal root ganglia (reactivation)	↑	[114]*
	Influenza virus A	i.n.; pneumonitis	↓	[32]†
	Chlamydia trachomatis (MoPn strain)	i.vg.; vaginal infection	–	[115,116]†
		i.vg.; mouse pneumonia (spleen infection)	↑	[117]†
	Chlamydia pneumoniae	i.n.; pneumonitis	↑	[118]†
	Helicobacter pylori	p.o.; chronic gastritis	–	[58]†
	Listeria monocytogenes (EGD 3B or ATCC 10403 strains)	i.v. or i.p.; listeriosis (liver, spleen)	↑	[119]† [30••]†
	Mycobacterium tuberculosis	i.v.; tuberculosis (lung, liver, spleen)	↑	[120]†
	Mycobacterium avium	i.v.; systemic infection (lung, spleen, liver)	– or ↓	[121,122]†
	Mycoplasma pulmonis (UAB CT strain)	i.n.; pneumonia	↑	[40]†
	Salmonella typhimurium (14028 strain)	i.p.; systemic infection	↑	[30••]†
	Salmonella typhimurium (recBC mutant)‡	i.p.; systemic infection	–	[30••]†
	Shigella flexneri	i.n.; bronchopulmonary infection	–	[123]†
	Staphylococcus aureus (TSST-1+ LS-1 strain)	i.v.; septic arthritis	↑	[124]*
	Leishmania major	s.c.; cutaneous leishmaniasis	↑ (non-healing) ↑ (healing)	[13•]†,[33]* [34•]*
	Leishmania donovani	i.v.; visceral leishmaniasis	↑	[125•]†
	Plasmodium berghei ANKA	i.v.; murine cerebral malaria	–	[126]†
	Plasmodium berghei XAT	i.v.; blood-stage malaria	–	[127]†
	Trypanosoma cruzi (Tulahuen strain)	i.p.; experimental Chagas' disease (spleen, liver, heart)	↑	[128]†
	Trypanosoma brucei	i.p.; experimental sleeping sickness	↓ or –	[129]*;[130]†
	Toxoplasma gondii	i.p. or p.o.; cerebral toxoplasmosis	↑	[131]†
		p.o.; inflammation of small intestine and liver	↓	[132]†
	Schistosoma mansoni	p.c.; immunized NOS2$^{+/+}$ or NOS2$^{-/-}$ mice challenged with live cercariae	(↑)§	[133]† [134]*
gp47^{phox}	Commensal *Staphylococci* and fungi	Spontaneous development of lethal deep infections (CGD mouse model)	↑	[135]
	Mycobacterium avium	Mycobacteriosis (spleen and lung)	–	[73]
	Listeria monocytogenes	Listeriosis (liver, spleen)	↑	[136]
gp91^{phox}	Commensal bacteria	CGD mouse model	↑	[137]
	Listeria monocytogenes	i.p.; listeriosis (liver, spleen)	↑ (day 2) – (day 3 or 6)	[30••,138]
	Salmonella typhimurium (14028 strain)	i.p.; systemic infection	↑	[30••,50]
	Salmonella typhimurium (recBC mutant)‡	i.p.; systemic infection	↑	[30••]
	Leishmania donovani	i.v.; visceral leishmaniasis	↑ (but healing)	[125•]
	Aspergillus fumigatus	i.t.; pulmonary aspergillosis	↑ (lethal)	[74]
Rac2	*Aspergillus fumigatus*	i.v.; disseminated aspergillosis (kidney, brain)	↑	[139]
NOS2 plus gp91^{phox}	*Listeria monocytogenes* (ATCC 10403 strain)	i.p.; listeriosis (liver, spleen)	– (50%) or ↑ (50%)	[30••]†
	Salmonella typhimurium (14028 strain)	i.p.; systemic infection	↑	[30••]†
	Salmonella typhimurium (recBC mutant)‡	i.p.; systemic infection	↑	[30••]†
MPO	*Staphylococcus aureus*	i.p.; peritonitis	–	[43]
	Candida albicans	i.t., pneumonia; i.p., disseminated candidiasis	↑ ↑	[43]

*In these studies NOS2-mutant mice were used, in which the intended deletion of exon 1 to 5 of the NOS2 gene had not occurred, and an alternative mRNA transcript of NOS2 was expressed [33] that later turned out to express reduced, but clearly functional, levels of NOS2 [34•]. †In these studies NOS2-deficient mice were used, in which the proximal 585 basepairs of the NOS2 promotor and exon 1 to 4 of the NOS2 gene were deleted and no NOS2 mRNA, protein or activity was detectable. ‡recBC mutants of *S. typhimurium* have a strongly reduced ability for DNA repair after oxidative damage. §The protective effect of vaccination is reduced in NOS2$^{-/-}$, compared with NOS2$^{+/+}$ mice. CGD, chronic granulomatous disease; i.n., intranasal; i.p., intraperitoneal; i.t., intratracheal; i.v., intravenous; i.vg., intravaginal; p.c., percutaneous; p.o., per os.

Table 2

Examples of NO as a signalling molecule in immunity.

Cell population	Stimulus	Source of NO	Phenotypic effect of NO	Molecular effect and target of NO	Reference
Mouse macrophages (ANA-1)	Mitogen (ConA)	Endogenous (NOS2)	Reduced expression of NF-κB-dependent genes (e.g. *NOS2* and *M-CSF*)	Inhibition of NF-κB p50 DNA binding by S-nitrosylation of p50	[103•]
Mouse macrophages (RAW264.7)	IFN-γ + LPS	Endogenous (NOS2) or NO donor (SNAP)	Inhibition of IL-1β and IL-18 release*	Inhibition of caspase-1 and caspase-1-mediated cleavage of pro-IL-1β and pro-IL-18	[102•]
Primary mouse T lymphocytes	Mitogen (ConA)	Mouse alveolar or peritoneal macrophages or NO donor (SNAP)	Inhibition of T cell proliferation	Inhibition of Jak3 and Stat5 tyrosine phosphorylation	[99]
Human Burkitt's lymphoma (10C9) or T cell leukemia lines (Jurkat, CEM)	Anti-Fas	Endogenous (NOS2) or NO donor (SNAP)	n.a.	Inhibition of caspase-3 by S-nitrosylation; anti-Fas leads to caspase-3 denitrosylation	[69••]
Mouse pro-B cell-line (Ba/F3)	IL-3	NO gas or NO donor (>1 mM DETA-NO)	Inhibition of proliferation	Inhibition of Jak2 kinase activity	[98]
Mouse NK cells (cell line or IL-2-expanded) primary NK cells)	IL-12	Endogenous (NOS2) or NO donor (1–100 μM SNAP or DETA-NO)	Prerequisite for IL-12-induced IFN-γ production*	Cofactor for Tyk2 kinase activity	[31••]
Murine endothelial cell line (TC10)	TNF-α	NO donor (glycerol-trinitrate)	Enhanced (25–250 μM) or diminished (>500 μM) DNA-binding of NF-κB	Inhibition of IκB-kinase-α	[100•]

*Study also presents evidence for a similar effect of NO *in vivo*. DETA-NO, diethylenetriamine nitric oxide; M-CSF, macrophage-colony-stimulating factor; n.a., not analysed; SNAP, S-nitroso-N-acetylpenicillamine.

or inhibitory cytokines (e.g. IL-4, IL-10, IL-13 and transforming growth factor β [TGF-β]) [4,5•]. These are produced by alternative populations of Th cells (Th1 or Th2), which therefore strongly affect the degree of NOS2 expression [10•]. The regulation of NOS2 can occur on multiple levels: NOS2 gene transcription, mRNA stability, mRNA translation and protein stability; the availability of substrates, cofactors and endogenous substrate analogues acting as NOS inhibitors; and the feedback effects of the end product (NO). More than 30 cytokines or cytokine-like factors have been described to date that increase or inhibit the expression of NOS2 activity in cells participating in the immune response: macrophages; microglia; Kupffer cells; neutrophils; eosinophils; mast cells; dendritic cells; natural killer (NK) cells; certain T cell and B cell lines; keratinocytes; fibroblasts; endothelial and epithelial cells; and vascular smooth muscle cells. Some cytokines —such as IFN-α/β, IL-4 or IL-10 — can exert bimodal effects on the production of NO by macrophages (reviewed in [5•]). IFN-α/β, which on their own are unable to induce NOS2 except in human monocytes [11,12], synergize with LPS or *Leishmania major* for the induction of NOS2 under costimulation conditions but have an inhibitory effect when added to rodent macrophages prior to the second stimulus (IFN-γ, LPS or *L. major*) ([13•,14•]; C Bogdan, unpublished data). In the latter situation, IFN-α/β have been shown to inhibit the activation of NF-κB [14•] or Stat1α (C Bogdan, unpublished data), two transcription factors that are critical for the induction of NOS.

Cell surface receptors

NOS2 is regulated by crosslinking of cell-surface receptors such as CD23 (Fcγ receptor IIb) [5•] or CD8 (α- or β-chain) [15]. Crosslinking of CD23 or CD8 leads to induction of NOS2 in human monocytes or rat macrophages, respectively. The adenosine receptor A_{2B}, which is induced by IFN-γ, in contrast mediates a feedback inhibitory effect on the expression of NOS2 mRNA in mouse macrophages upon binding of adenosine or adenosine analogues [16•].

Microbial products

Another group of NOS2 regulators are products of viruses, bacteria, protozoa or fungi. LPS is the prototypic example; it stimulates rodent macrophages to express NOS2, at least partly via induction of endogenous IFN-α/β [17]. LPS usually synergizes with IFN-γ for the induction of NOS2 but can also have antagonistic effects, depending on the concentration and sequence of the stimulation (reviewed in [5•]). Similar to LPS, other microbial lipoproteins (e.g. the 19 kDa *Mycobacterium tuberculosis* lipoprotein or *Borrelia burgdorferi* OspA [18•]) and bacterial exotoxins (e.g. pneumolysin of *Streptococcus pneumoniae* or tracheal cytotoxin of *Bordetella pertussis*) induced the expression of NOS2 activity in macrophages and other cell types. In some cases their

action was dependent on the presence of LPS or of IFN-γ [19,20•] and was strongly associated with deleterious rather than host-protective effects [20•].

Several infectious pathogens (e.g. *Leishmania* spp. or *Candida albicans*) were reported to inhibit the expression of NOS2 in macrophages [21•,22]; this might contribute to the very limited expression of NOS2 early during infection [13•] and facilitate the initial survival of the parasites in the host. In the case of *L. major*, the suppression appears to be mediated by glycoinositolphospholipids of the parasites [5•]. For *L. donovani*, it was reported that the parasites might activate cellular phosphotyrosine phosphatases and thereby impair the signalling cascade that otherwise leads to the expression of NOS2 [21•].

Other regulatory factors

A recent study showed that the human heat shock protein (hsp)60 has some capacity to stimulate the J774 mouse macrophage line for the production of NO but the (patho)physiologic relevance of this finding is presently unclear despite the known release of intracellular hsp60 during cell necrosis [23•]. In addition to the macromolecular regulators of NOS2, several small inorganic molecules have been described to affect the expression and/or activity of NOS2. Depletion of non-ferritin-bound iron (Fe^{2+}) [24], a decrease of oxygen tension [25] or low environmental pH [26] have all been found to enhance NOS2 gene transcription. This might accelerate the resolution of infections with intracellular pathogens but could also promote tissue damage during chronic inflammatory processes. Finally, the expression of NOS2 is also positively or negatively affected by a number of drugs (e.g. glucocorticoids, aspirin, taxol, tetracyclines or antifungal imidazoles) that are used for the treatment of autoimmune, infectious or malignant diseases [5•,27–29]. To date it is unclear to what extent the induction or suppression of NOS2 promotes or counteracts the therapeutic value of these compounds in humans.

Antimicrobial activity of RNIs and ROIs

In vivo models

The antimicrobial activity of ROIs and RNIs has been most convincingly demonstrated by the use of transgenic mice deficient for one or several antimicrobial effector pathways. These studies have been informative in several respects. First, they showed that a broad spectrum of infectious agents (ranging from viruses to helminths) is directly or indirectly controlled by RNIs and/or ROIs *in vivo*; importantly, these studies did not rely on ROI or RNI inhibitors or scavengers with questionable activity or specificity (Table 1). Second, *Salmonella typhimurium* or *Listeria monocytogenes* infection of macrophages from mice lacking both NADPH oxidase and NOS2 provided evidence for an additional mechanism that impedes the replication of these bacteria [30••]. Third, in some models NOS2-derived NO was produced and functional already during the very early (innate) phase of infection; this demonstrates that the expression of NOS2 is not restricted to the adaptive phase of the immune response, when IFN-γ-producing Th cells prevail [13•,31••]. Fourth, the analysis of $NOS2^{-/-}$ mice revealed that NOS2-derived NO is dispensable for the control of several pathogens (Table 1) or might be even counterprotective [5•,32]. Some of these results, however, will require re-evaluation because they were obtained with a strain of NOS2-mutant mice [33] in which the NOS2 gene was not deleted and an alternative NOS2 transcript was expressed. Although these mice were originally reported to lack NOS2 activity and to develop progressive leishmaniasis upon infection with *L. major* [33], a recent analysis by the same group demonstrated residual NOS2 activity in these mice that was functional and sufficient to heal an infection with *L. major* [34•]. These data disprove the recently proposed existence of an antimicrobial mechanism that even in the absence of NOS2 is sufficient to control *L. major in vivo* [35].

Mechanisms of antimicrobial activity

In the past year several studies have provided important new insights into the mechanisms of antimicrobial function of RNIs and ROIs (for previous reviews, see [36,37]). Saura *et al.* [38••] identified (in Coxsackie-virus-infected epithelial cells) the viral cysteine protease 3C as the target of macrophage-derived NO, which inactivates the protease via S-nitrosylation of the cysteine residue in the active site and thereby interrupts the viral life cycle; this study provided strong evidence for a direct antiviral effect of NO. Although NO donors could substitute for cytokine-activated macrophages in this system, this does not imply that the ·NO radical is always the active molecular species. In macrophages cocultured with the extracellular protozoon *Trypanosoma musculi*, the NOS2-dependent killing of the parasites was strictly dependent on the presence of albumin; S-nitrosylated albumin, but not free NO, exerted the antiparasitic effect [39]. In other systems, peroxinitrite ($ONOO^-$) rather than NO or O_2^- turned out to account for the killing of the infectious pathogen by macrophages [40]. Although NO and O_2^- are usually derived from host cells, significant amounts of O_2^- can also be produced by certain bacteria (e.g. *Helicobacter pylori*); such O_2^-, upon reaction with host-cell-derived NO and formation of peroxinitrite (see Figure 1), blocks microbial respiration [41].

Another form of agonistic interaction between RNIs and ROIs was recently reported by Andonegui *et al.* [37], who found that long-term treatment (8–18 hours) of human neutrophils with NO donors leads to an increased production of O_2^- and H_2O_2. However, previous studies have reached the opposite conclusion — they showed inhibition of NADPH oxidase activity after short-term exposure (2 minutes) to NO (see [42] and references therein).

Although the actual level to which the MPO/H_2O_2/Cl^- pathway contributes to the defense against microbial pathogens *in vivo* has been controversial throughout the years [1],

recent *in vitro* and *in vivo* studies have refueled ideas supporting its biological significance [43,44]. Experiments with $MPO^{-/-}$ mice (Table 1) confirmed earlier clinical experience with MPO-deficient patients, some of whom developed major systemic (fungal) infections — in particular with *C. albicans* (see [45] and references therein). Furthermore, activated human neutrophils were shown to use MPO for conversion of nitrite (NO_2^-) into the oxidants nitryl chloride (NO_2Cl) and nitrogen dioxide ($\cdot NO_2$) that can cause tyrosine nitration and chlorination of target molecules [46••] (Figure 1). NO_2^--dependent tyrosine nitration reactions also occur in human eosinophils from MPO-deficient individuals, which possibly explains why most of these patients are not at an increased risk of infection [47]. *In vivo*, the nitrite might be supplied by activated macrophages in the vicinity of the neutrophils or eosinophils, or might originate from the primary granules of neutrophils, in which MPO was found to colocalize with NOS2 [48]. The convergence of the NOS2 and MPO pathways might represent a novel mechanism of the antimicrobial machinery of granulocytes.

Resistance to ROIs and RNIs

Phenotypic resistance to ROIs has been observed in a number of bacteria (e.g. koagulase-negative staphylococci, *Escherichia coli*, *Salmonella typhimurium* and *M. tuberculosis*) and involves the expression of O_2^-- and H_2O_2-scavenging enzymes such as SOD, catalase and glutathione reductase. In *M. tuberculosis*, expression of even small amounts of catalase activity strongly protected the bacilli against oxidative killing by H_2O_2 [49]. In *S. typhimurium*, periplasmic copper/zinc-SOD detoxified O_2^- and thereby most probably impeded the formation of $ONOO^-$, thus protecting the bacteria against O_2^- and NO [50]. In *E. coli*, exposure to O_2^- activates a distinct set of antioxidant genes that are under the control of the SoxRS regulon (e.g. genes for manganese-containing SOD [the gene is called *sodA*], oxidative DNA repair enzyme endonuclease IV [*nfo*] and glucose-6-phosphate dehydrogenase [*zwf*]). Similarly, H_2O_2 stimulates the thiol-containing transcriptional activator OxyR which controls the expression of genes for catalase/hydroperoxidase (*katG*), glutathion reductase (*gorA*) and alkyl hydroperoxidase reductase subunit C (*ahpC*) in *E. coli* and *S. typhimurium*. Interestingly, both the SoxRS and the OxyR regulon are also responsive to NO or S-nitrosothiols, suggesting a common defense strategy against nitrosative and oxidative stress (see [51,52••] and references therein; see also Figure 1).

Recently, several genes and proteins were identified that confer protection against RNIs in *E. coli*, *S. typhimurium* and/or *M. tuberculosis* as shown by gene overexpression or by complementation of mutants. These include four genes products in particular: first, flavohemoglobin (*hmp*), which exhibits an NO dioxygenase activity (i.e. it detoxifies NO to NO_3^-) and is induced by NO via inactivation of an iron-dependent repressor [53–55]; second, alkyl hydroperoxidase reductase subunit C (*ahpC*), which is regulated by OxyR but whose mechanism of protection against RNIs is unknown [52••]; third, the bifunctional enzyme aspartokinase II-homoserine dehydrogenase II (*metL*), which is critical for the synthesis of homocysteine — an antagonist of S-nitrosothiols in *S. typhimurium* [56]; and, fourth, two novel genes (*noxR1* and *noxR3*) that were isolated from *M. tuberculosis*, protect against both RNIs and ROIs by an as-yet unknown mechanism and are not expressed in nonpathogenic or opportunistic mycobacteria (see [57] and references therein). In *H. pylori*, CO_2/HCO_3^- produced by the bacterial urease might protect the microbe against peroxynitrite [58]. Thus, pathogenic bacteria are most probably equipped with a whole array of mechanisms that mediate resistance to oxidative and nitrosative effector molecules of the host (Figure 1).

Although studied to a much smaller extent, antioxidant mechanisms also exist in protozoa (reviewed in [59]) and fungi (see [60] and references therein) and are similarly critical for the survival of the parasites in the host.

Immunoregulation by RNIs and ROIs

The first immunoregulatory activity that could be assigned to ROIs and RNIs was their ability to inhibit the proliferation of lymphocytes. Together with prostaglandins, the production of NO by NOS2 and of ROIs by the NADPH oxidase are the key mechanisms by which 'suppressor macrophages' impair the proliferative response of T lymphocytes to antigens or mitogens. This ROI/RNI-mediated inhibition of T cell proliferation at least partially accounts for the immunosuppressed state seen in certain infectious diseases, malignancies and graft-versus-host reactions but might also serve to control inflammatory processes or to delete autoreactive T cells and thereby fulfils host-protective functions (reviewed in [5•]). Recently, a number of molecularly defined mechanisms have emerged by which ROIs and RNIs modulate the immune response.

Cytokine production

To date, (NOS2-derived) NO is known to affect the production of more than 20 cytokines (including IL-1, IL-6, IL-8, IL-10, IL-12, IFN-γ, TNF-α and TGF-β) by various immune cells (e.g. macrophages, T lymphocytes, NK cells and endothelial cells) as assessed by the use of NOS inhibitors, $NOS2^{-/-}$ mice and/or NO donors (reviewed in [5•]). Although in most cases the pathway that is subject to regulation by NO remains to be identified, a number of signal-transduction molecules are already known to be positively or negatively regulated by NO (see below and Table 2). For several cytokines, conflicting results have been reported as to the effect of NO; in some cases this might reflect the use of different cell populations. The production of IL-12 (p40 or p70) by macrophages (for example) was facilitated, inhibited or remained unaltered by NOS2-derived NO [13•,61–63]. In rat and mouse NK cells, NOS2-derived NO was partially or absolutely required for the production of IFN-γ in response to IL-2 or IL-12 (respectively) whereas in human NK cells expression of NOS2 inhibited the subsequent secretion of

IFN-γ [13•,31••,64,65•]. NOS2-dependent production of IFN-γ by NK cells has also been observed during the innate phase of the immune response of mice infected with *L. major* [13•,31••].

Whether NO also regulates the cytokine production of Th cells has received considerable attention during the past years because it might be relevant for the development of a Th1 response compared with a Th2 response. The published results have not been uniform. Controversy exists with respect to three aspects: first, the level of expression of NOS2 by T lymphocytes [66–68,69••]; second, the effect of exogenous NO on the cytokine production by primary T cells [34•,68,70]; and, third, with respect to a (possibly selective) effect of exogenous NO on the cytokine release by established Th1 (compared with Th2) lines and clones [34•,66,68,71,72]. Differences in the cell type (primary compared with cloned, mouse compared with human, transformed compared with nontransformed), the donors (e.g. agents releasing ·NO, NO^+, ·NO plus O_2^- or ·NO plus cyanide) and their concentrations, and the stimulation conditions (mitogen compared with antigen-presenting cells [APCs] plus antigen) are parameters that are likely to determine whether NO stimulates, inhibits or does not alter T cell differentiation and function. This is underlined by a recent study which demonstrates that low doses (1–10 μM) of S-nitrosopenicillamin upregulated, whereas higher concentrations (100 μM) suppressed, the production of IFN-γ by primary T cells cultured with APCs, antigen and IL-12. The cytokine response of established T cell lines, in contrast, was largely inert in response to the NO donor [34•]. As the actual concentrations of NOS2-derived NO *in situ* are variable and not predictable, it is impossible to make a definitive statement on the effect of NO on Th1/Th2 development. Accordingly, NOS2 deficiency in mice was found to be associated with an enhanced, an unaltered or a diminished Th1 response in different infectious disease models (reviewed in [5•]).

Compared with the results on RNIs there is much less evidence for a cytokine-regulatory function of ROIs *in vivo*. This might be due to the fact that there is more redundancy in the production of ROIs compared with RNIs. In mice lacking the $gp47^{phox}$ protein that were infected with *Mycobacterium avium* or *Schistosoma mansoni* eggs, there was no difference in the development of the Th cell cytokine response when compared with wild-type mice [73]. In mice deficient for the $gp91^{phox}$ and challenged with sterile *Aspergillus fumigatus* hyphal cell walls, the expression of IL-1β, TNF-α, the chemokines KC and JE, and of TGF-β were significantly elevated in the lungs compared with wild-type controls [74].

Apoptosis

Native NO or S-nitrosothiols can induce or block apoptosis in many cell types. Proapoptotic effects (e.g. in macrophages or T lymphocytes) were observed with high (100–200 μM) concentrations of exogenous NO and were associated with the accumulation of p53, which at least in part is due to an NO-mediated inhibition of the degradation of polyubiquitinated p53 by the 26S proteasome (see [75•] and references therein). Peroxynitrite ($ONOO^-$) primed T lymphocytes to undergo apoptotic cell death after subsequent activation by phorbol esters or anti-CD3. The effect of $ONOO^-$ is likely to involve an inhibition of protein tyrosine phosphorylation via nitration of tyrosine residues [76]. Other mechanisms by which (endogenous) NO might promote apoptotic events include the upregulation of the cell surface receptor Fas (CD95) or its ligand (FasL, CD95L) [77,78•]. NO, however, can also induce apoptosis without measurable modulation of Fas/FasL [79]. There is considerable evidence that NOS2-derived NO contributes to the deletion of double-positive T lymphocytes in the thymus and to the elimination of autoreactive T cells (see [80,81•] and references therein).

In the immune system, an antiapoptotic function of endogenous NO generated by NOS2 has been observed in B cell lines, macrophages, eosinophils and T lymphocytes (reviewed in [5•]). In mouse macrophages, nontoxic concentrations of NO lead to the activation of the transcription factors NF-κB and AP-1 and the subsequent expression of cyclooxygenase-2 which protected the macrophages against apoptosis [82]. In human eosinophils, NO inhibited Fas-receptor-induced activation of Jun kinase and the proteolysis of lamin B1 — a protein that is known to rapidly decrease in apoptotic cells [83]. In resting human B and T cell lines, the unprocessed (zymogen) form of caspase-3, one of the downstream caspases executing apoptosis, was found to be S-nitrosylated on its active-site cysteine. Upon activation of the cells via crosslinking of Fas, the caspase-3 zymogen was cleaved into its active subunits and the active-site thiol was denitrosylated [69••]. As NO inhibits the activity of caspase-3 in cell-free systems (via S-nitrosylation) as well as in intact cells (see [69••,84] and references therein), these data suggest that S-nitrosylation helps to maintain the proenzyme in an inactive form and that denitrosylation of caspase-3 is an integral part of the Fas apoptotic pathway. Similar observations have also been made with hepatocytes, in which NO protected against TNF-α-induced activation of caspase-3 and -8 and apoptotic cell death [85]. This might explain the impaired liver regeneration after injury in mice lacking NOS2 activity [86]. Despite this cell-protective function of NO, it is important to bear in mind that NO, while inhibiting apoptotic events, can still cause necrotic cell death in the same cells [87]. The reversion of blocked apoptosis into necrosis presumably requires high concentrations of NO, is therefore most likely to occur during strong cytokine responses and might account for the tissue damage seen in chronic inflammatory processes.

Similar to RNIs, reactive oxygen species were reported to induce or suppress apoptosis of various cells of the immune system. Human NK cells were readily killed by H_2O_2 produced by monocytes whereas human T cells were two- to five-times more resistant to H_2O_2-induced apoptosis [88]. In mouse macrophages, endogenously pro-

duced O_2^- was shown to contribute to resistance to NO-mediated apoptosis (presumably via scavenging of NO) — indicating that the balance between intracellularly produced ROIs and RNIs could determine macrophage apoptosis/survival (see [89] and references therein). In human neutrophils stimulated with a protein kinase C activator (phorbol myristate acetate), NADPH oxidase activity completely suppressed the rapid induction of caspase activity but promoted the early externalization of phosphatidyl serine (which serves as a recognition signal of apoptotic cells by macrophages). Thus ROIs, similar to endogenous NO (see above), might prevent caspases from functioning but at the same time an oxidant-dependent pathway is used to mediate exposure of phosphatidylserine, cell death and subsequent neutrophil clearance. The existence of two apoptosis pathways might explain why neutrophils from NADPH-oxidase-deficient patients can still undergo apoptosis spontaneously or in response to Fas-triggering (see [90] and references therein).

Another example for the existence of a caspase-independent pathway of apoptosis, mediated by endogenous (mitochondrial?) ROIs, has recently been reported for primary T lymphocytes isolated from mice after treatment with superantigens. *In vitro*, a large percentage of these cells spontaneously developed signs of apoptosis (exposed phosphatidylserine, DNA fragmentation, nuclear condensation and loss of mitochondrial membrane potential) and died (due to acquisition of membrane permeability) unless they were cultured in the presence of a mimetic of superoxide dismutase — Mn (III) tetrakis (5,10,15,20-benzoic acid) porphyrin (MnTBAP). Caspase inhibitors, in contrast, only prevented DNA degradation but not cell death. The ROI-mediated apoptosis did not require Fas- or TNF-receptor signalling [91••]. Thus, based on the primary apoptotic signal, T cell apoptosis is triggered either by cell surface death-receptor-dependent caspase activation or by caspase-independent ROI-mediated mitochondrial damage. Both pathways will finally result in loss of mitochondrial membrane potential, DNA degradation and intracellular disintegration.

Signalling

In vitro experiments with purified target proteins or intact cells have identified RNIs or ROIs as regulators of a broad spectrum of signalling pathways. Examples include ion channels, G proteins (e.g. the proto-oncogene $p21^{ras}$), protein tyrosine kinases (e.g. Fyn and other members of the src family of tyrosine kinases), protein tyrosine phosphatases, Janus kinases (Jak1, Jak2, Jak3 and Tyk2), mitogen-activated protein kinases (extracellular signal-regulated kinases, Jun N-terminal kinase, stress-activated protein kinase), caspases and various transcription factors (e.g. NF-κB, AP-1, Sp1 and c-Jun) (reviewed in [5•,92–95]). Many of these studies have relied on the use of exogenous sources of RNIs or ROIs (frequently at concentrations which are not compatible with life) and therefore did not allow claims of a physiological role of RNIs or ROIs in this respect.

More recently, however, several reports convincingly demonstrated a signalling role of small quantities of exogenous or endogenously produced RNIs [31••,69••,96,97•,98,99,100•,101,102•,103•] (see Table 2). Molecular mechanisms by which NO inhibits or stimulates the action of transcription factors and other members of signal-transduction pathways include S-nitrosylation, S-glutathionylation, disruption of zinc fingers and the formation of iron–nitrosyl complexes (in the case of heme proteins) (reviewed in [5•]). As to the role of ROIs, there is evidence that H_2O_2 (or an oxygen species generated through Fenton chemistry [Figure 1]) facilitates the tyrosine-phosphorylation of Stat3 and Jak2 [94,95]. Endogenous or exogenous ROIs have been implicated in the activation of NF-κB in a number of cell types but chronic exposure to oxidative stress (100 μM H_2O_2) inhibited the phosphorylation and degradation of IκBα and thereby impeded NF-κB-dependent transcriptional activity in T lymphocytes (see [104] and references therein).

Constitutive NO synthases and the immune system

The expression of the constitutive NO synthases is not restricted to neurones or endothelial cells, as is suggested by the commonly used acronyms ncNOS and ecNOS. Ca^{2+}-dependent NO synthase activity was found in rat thymocytes [105] and NOS1 or NOS3 was present in human monocytes/macrophages and in B and T lymphocytes (see [69••,106] and references therein). NOS1 or NOS3 can also assume typical immunological functions previously assigned to NOS2, such as the induction of apoptotic cell death [78•] and the control of viruses [107,108]. Similarly, a recently described Ca^{2+}-dependent NOS isoform of plants was shown to exert an antibacterial effect against *Pseudomonas syringae* that was inhibitable by cNOS inhibitors [109].

Conclusions

Even in modern textbooks on immunology a frequently conveyed message is that ROIs as products of neutrophils primarily act as antimicrobial effector molecules in the innate defense against extracellular bacteria whereas RNIs are mainly generated by macrophages in the context of a specific type 1 Th cell response, where they contribute to the control of intracellular pathogens. This review intended to illustrate that the functions assumed by ROIs and RNIs in the immune system are far more varied. To date, there is no doubt that both groups of inorganic intermediates are integral parts of immunologically important signalling pathways, regulate cytokine responses and cell survival and contribute to tissue damage as seen in autoimmune diseases or other forms of chronic inflammatory processes. There is also no reason to assume that these effects are restricted to animal models and do not occur in humans. In particular, to deny the importance of the production of NO by human monocytes, macrophages and other human cells or to dismiss it as functionally irrelevant (on the grounds that the

levels are often considerably lower compared with rodent cells) is without scientific basis: human cells (including blood monocytes) do express NOS2 activity — as has been shown in many different diseases [110•,111•] — and even small amounts of NO can exert immunoregulatory effects, as reviewed above. One major task for the future, however, will be to delineate the actual function(s) of the NO produced by human cells in the various disease states.

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