Nitric oxide and the immune response

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During the past two decades, nitric oxide (NO) has been recognized as one of the most versatile players in the immune system. It is involved in the pathogenesis and control of infectious diseases, tumors, autoimmune processes and chronic degenerative diseases. Because of its variety of reaction partners (DNA, proteins, low–molecular weight thiols, prosthetic groups, reactive oxygen intermediates), its widespread production (by three different NO synthases (NOS) and the fact that its activity is strongly influenced by its concentration, NO continues to surprise and perplex immunologists. Today, there is no simple, uniform picture of the function of NO in the immune system. Protective and toxic effects of NO are frequently seen in parallel. Its striking inter- and intracellular signaling capacity makes it extremely difficult to predict the effect of NOS inhibitors and NO donors, which still hampers therapeutic applications.

When nitric oxide (NO) formally entered the immunology scene, between 1985 and 1990, its role in the immune system was simply defined: NO is a product of macrophages activated by cytokines, microbial compounds or both, is derived from the amino acid L-arginine by the enzymatic activity of inducible nitric oxide synthase (iNOS or NOS2) and functions as a tumoricidal and antimicrobial molecule *in vitro* and *in vivo*[1]. (Unless otherwise specified, the term nitric oxide—NO without a dot for the unpaired electron—is used here collectively for all reactive nitrogen intermediates (RNI) that have been invoked as either immediate products of the NOS reaction (•NO radical, NO^-, NO^+) or their adducts or conversion products. The latter category includes NO_2, NO_2^-, NO_3^-, N_2O_3, N_2O_4, S-nitrosothiols (S-NO), peroxynitrite ($ONOO^-$) and nitrosyl–metal complexes.)

Although this basic definition is still accepted, during the past decade it has been recognized that NO plays many more roles in the immune system (**Table 1** and below) as well as in other organ systems. There are a number of causes for this. First, in addition to macrophages, a large number of other immune-system cells produce and respond to NO.

Second, and contrary to previous views, all known isoforms of NO synthase—neuronal NOS (nNOS, or NOS1), iNOS and endothelial NOS (eNOS, or NOS3)—operate in the immune system. (The nNOS and eNOS isoforms are also known collectively as constitutive NOS (cNOS), because unlike iNOS they usually exist as constitutively expressed proteins in the cell and are primarily regulated by Ca^{2+} fluxes and subsequent binding of calmodulin[2]. Their expression is not restricted to neurons or endothelial cells.) Although the three isoforms catalyze the same reaction, the conversion of L-arginine and molecular oxygen to N^{ω}-hydroxy-L-arginine and further to citrulline and NO, they differ with respect to their regulation, the amplitude and duration of the production of NO, and their cellular and tissue distribution[2,3]. As another level of complexity, NOS activity is determined by several mechanisms, many controlled by immunological stimuli (as discussed below).

Third, the activity of NO is not restricted to the site of its production. As an uncharged gas, •NO radicals are highly diffusible. Low-molecular weight S-nitrosothiols (such as *S*-nitrosoglutathione), S-nitrosylated proteins, and nitrosyl-metal complexes can function as long-distance NO vehicles[4], which liberate NO either spontaneously or after cleavage by ectoenzymes found on cells such as T and B lymphocytes[5]. Furthermore, N^{ω}-hydroxy-L-arginine, which is secreted by cells and detectable in the plasma, can be oxidized to citrulline and NO by a number of hemoproteins (such as peroxidases and cytochrome P450) as well as superoxide anions[6]. Likewise, circulating nitrite (NO_2^-), a stable product of the NOS reaction, can be reduced to •NO under mildly acidic conditions and is a substrate of the peroxidase pathways of neutrophils and eosinophils that can lead to the formation of novel NO-derived oxidants at distant sites[7,8]. Therefore, NOS-negative immune cells can both produce NO and become targets of NO action.

Fourth, in contrast to cytokines, the interaction of NO is not restricted to a single defined receptor; rather, it can react with other inorganic molecules (such as oxygen, superoxide or transition metals), structures in DNA (pyrimidine bases), prosthetic groups (such as heme) or proteins (leading to S-nitrosylation of thiol groups, nitration of tyrosine residues or disruption of metal–sulfide clusters such as zinc-finger domains or iron–sulfide complexes)[9]. Considering that many of the targets of NO are themselves regulatory molecules (for example, transcription factors and components of various signaling cascades)[10], it is evident that NO frequently exerts heterogeneous and diverse phenotypic effects.

This review summarizes studies published during the past two years that provide novel insights into the role of NO in the immune system. It focuses particularly on (i) the cellular expression and possible function of the different NOS isoforms in immune cells other than macrophages (ii) post-translational mechanisms of regulation of NOS activity (iii) results of gene-chip approaches to assess the signaling capacity of NO (iv) the role of NO in the thymus (v) indirect antimicrobial effects of the iNOS pathway (vi) stage- and organ-specific activities of NO during infectious diseases, and (vii) the impact of iNOS and cNOS in autoimmune processes. For discussion of earlier studies and detailed discussion of other functions of NO in the immune system, the reader is referred to previous reviews[2,11–17].

NO production in the immune system

Generation of NO is a feature of genuine immune-system cells (dendritic cells, NK cells, mast cells and phagocytic cells including monocytes, macrophages, microglia, Kupffer cells, eosinophils, and neutrophils) as well as other cells involved in immune reactions (such as

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endothelial cells, epithelial cells, vascular smooth muscle cells, fibroblasts, keratinocytes, chondrocytes, hepatocytes, mesangial cells and Schwann cells)[17]. Either iNOS or eNOS have been found in macrophages, dendritic cells, and natural killer (NK) cells and in cell lines, clones, hybridomas and tumor cells of B or T cell origin (**Table 2**). Whether primary T or B lymphocytes express any of the NOS isoforms remains questionable. Some positive reports could not be confirmed in other settings[18–21] or relied solely on the detection of NOS mRNA by PCR (raising the possibility of false-positive results due to contaminating cells)[22]. Other reports did not corroborate indirect evidence (such as the effect of NOS inhibitors, detection of nitrotyrosine or immunocytochemical staining) by directly demonstrating the presence of the NOS protein (for example, by western blotting using cells from gene-targeted mice as controls)[23,24].

Mechanisms of regulation of NO production

The expression of iNOS is regulated by cytokines and determined primarily by the *de novo* synthesis and stability of iNOS mRNA and protein[2,25,26]. In contrast, nNOS and eNOS exist in the cell as preformed proteins whose activity is switched on by the elevation of intracellular Ca^{2+} concentrations and the binding of calmodulin in response to neurotransmitters or vasoactive substances[3]. Beyond this basic paradigm, additional levels of regulation exist for all three NOS isoforms that may operate during immune responses.

Activation of the iNOS gene promoter is an important mode of iNOS regulation by cytokines, which has been analyzed most thoroughly in mouse macrophages and in human hepatocyte and epithelial cell lines. The list of participating transcription factors includes NF-κB, AP-1, the signal transducer and activator of transcription (STAT)-1α, interferon

Table 1. Overview of immune-system NO function

Category	Producers of NO (examples)	Phenotypic effect of NO	Examples of underlying molecular mechanisms	Ref.
		Effector functions		
Antimicrobial activity	Macrophages, microglia, neutrophils, eosinophils, fibroblasts, endothelial cells, epithelial cells, astroglia	Killing or reduced replication of infectious agents (viruses, bacteria, protozoa, fungi, helminths)	•Direct effect of NO on the pathogen (see text) •Indirect effects of the NOS pathway (*e.g.*, reaction of NO with other effector molecules, arginine depletion; see text)	14,16,99
Anti-tumor activity	Macrophages, eosinophils	Killing or growth inhibition of tumor cells	•Inhibition of enzymes essential for tumor growth (*e.g.*, enzymes of the respiratory chain, *cis*-aconitase, ribonucleotide reductase arginase, ornithine decarboxylase) •Growth inhibition *via* iNOS-dependent depletion of arginine •Cell-cycle arrest (downregulation of cyclin D1) •Induction of apoptosis (by activation of caspases and accumulation of p53) •Sensitization of tumor cells for TNF-induced cytotoxicity	1,17,144,145,187
Tissue-damaging effect (immunopathology)	Macrophages, microglia, astroglia, keratinocytes, mesangial cells	Necrosis or fibrosis of the parenchyma	•Apoptosis of parenchymal cells •Degradation of extracellular matrix •Deposition of matrix, proliferation of mesenchymal cells •Influx of inflammatory cells via chemokine regulation	13,17,73
		Immunoregulatory functions		
Anti-inflammatory–immunosuppressive effect	Macrophages ('suppressor phenotype')	Inhibition of: •T cell proliferation •B cell proliferation •Antibody production by $CD5^+$ B cells •Autoreactive T and B cell diversification Inhibition of leukocyte recruitment (adhesion, extravasation, chemotaxis)	•Apoptosis of T cells or APCs •Downregulation of MHC class II, costimulatory molecules or cytokines •Disruption of signaling cascades and transcription factors •Inhibition of DNA synthesis •Downregulation of adhesion molecules or chemokines	5,10,17,77,116,135, 139–141,146,147
Modulation of the production and function of cytokines, chemokines, and growth factors (pro- or anti-inflammatory effects)	Macrophages T cells endothelial cells fibroblasts	Up- and downregulation, *e.g.*, of: •IL-1, IL-6, IL-8, IL-10, IL-12, IL-18, IFN-γ, TNF •TGF-β, G-CSF, M-CSF, VEGF, •MIP-1α, MIP-2, MCP-1	Modulation of •Signaling cascades (*e.g.* G-proteins, Jak, MAP kinases, caspases, protein phosphatases) •Transcription factors (*e.g.* NF-κB, Sp1, AP-1) •Proteins regulating mRNA stability or mRNA translation •Latent cytokine precursor complexes •Enzymes that process cytokine precursors	9,10,148–153
T helper cell deviation	*e.g.*, macrophages	•Induction and differentiation of T_H1 cells •Suppression of T_H1 (and T_H2) cell responses •Suppression of tolerogenic T cell responses	1. Possible stimulation of IL-12-mediated signaling 2. Suppression of IL-12 production	17,20,140,154

Table 2. Selected reports on the expression and function of NOS isoforms in phagocytes, dendritic cells, NK cells and T and B cell lines[a,b].

Cell type	Stimulus	NOS isoform[c]	Proposed function of NO	Ref.
		Macrophages		
Mouse or human mϕ	*e.g.*, IFN-γ + LPS; IFN-α/β; IL-4 plus anti-CD23	iNOS (R, P, A)	Antimicrobial activity; T cell suppression	17,116,147
Rat alveolar mϕ	None or lung surfactant	eNOS (P, A)	Anti-inflammatory effect?	155
Human promonocytic cells (U937)	sCD23 or anti-CD11b/c	eNOS (R, P, A)	?	156
		Dendritic cells		
Primary mouse LC	IFN-γ + LPS; IFN-γ + L. major	No iNOS mRNA detectable	n.a.	157
Primary mouse LC, LC line	LPS; IFN-γ + LPS	iNOS (R,P,A)	Proinflammatory effect?	158,159
Mouse BM-DC (mature)	IFN-γ + LPS; coculture with allogeneic T cells; anti-CD40	iNOS (R,P,A)	T cell growth ↓ DC apoptosis	160
Mouse BM-DC (immature)	IFN-γ + LPS	iNOS (A)	Microbial growth ↓	161
Mouse fetal skin-DC line	LPS, TNF or GM-CSF	iNOS (P, A)	?	162
Rat thymic DC	None; self-antigens, allo-antigens	iNOS (P, A)	Apoptosis of double-positive thymocytes?	86
		NK cells		
Mouse splenic NK cells, NK cell line	IL-2 ± IL-12 or IFN-α/β [d]	iNOS (R,P,A)	Tyk2 kinase ↑, IFN-γ release ↑ cytotoxicity ↑	113
Mouse uterine NK cells	gestation	iNOS (R, P)	Expression of perforin	163
Rat NK cells (blood, spleen)	IL-2	iNOS (R,P,A)	Cytotoxicity ↑, IFN-γ release ↑	164
Human blood NK cells	IL-12 +/or TNF	iNOS (R,P,A)	Cytotoxicity ↓, granzyme B expression ↓, IFN-γ release ↓	165
Human blood NK cells/lines	IL-2 + anti-CD16 or target cell contact	eNOS (R,P,A); no iNOS	Anti-apoptotic effect	166
		T cells		
Mouse T cell hybridoma	anti-CD3	nNOS (P, A)	Proapoptotic effect	24
Leukemic T cells (Jurkat)	HIV-1 infection	iNOS (R)	Viral replication	21
Human leukemic T cell lines[e]	None	iNOS (P); no iNOS (R)	Anti-apoptotic effect; n.a.	167;65
Human leukemic T cell lines[f], ATL cells	Infection with HTLV-I	iNOS (R,P,A)	?	65
Human leukemic T cell line (Jurkat)	SDF1α	(e)NOS (A)	Chemotactic response to SDF1α ↑	84
Human γδ T cell clones	IL-2, anti–T cell receptor	eNOS (P, A)	Anti-apoptotic effect	168
		B cells		
Human Burkitt´s lymphoma cells	None	iNOS (P)	Anti-apoptotic effect	167
Human B-CLL cells	None	iNOS (R, P, A)	Anti-apoptotic effect	169

[a]See text for data on primary T and B lymphocytes.
[b]Abbreviations: ATL, adult T cell leukemia; BM, bone marrow; CLL, chronic lymphocytic leukemia; DC, dendritic cells; LC, Langerhans cells; n.a., not applicable; PHA, phytohemagglutinin; SDF, stromal cell–derived factor.
[c]R, mRNA; P, protein; A, enzyme activity.
[d]In the presence of IL-18, the production of IFN-γ by NK cells remained unaltered in the absence of iNOS[113]. This might also explain why iNOS$^{-/-}$ mice developed normal NK cell activity after viral infections[170,171].
[e]Jurkat, H9, CEM.
[f]MT-1, SLB-1, C5/MJ

regulatory factor-1 (IRF-1), nuclear factor interleukin-6 (NF-IL-6) and the high-mobility group-I(Y) protein[27–31]. Depending on the cytokine or microbial stimulus and the cell type, different upstream signaling pathways are involved that promote (for example, Janus kinases Jak1, Jak2 and tyk2; Raf-1 protein kinase; mitogen-activated protein kinases p38, Erk1/2 and JNK; protein kinase C; protein phosphatases 1 and 2A) or inhibit (for example, phosphoinositide-3-kinase, protein tyrosine phosphatases) iNOS expression[17,32–35]. NO itself exerts a biphasic effect on the transcription of iNOS. Low concentrations of NO (such as occur at the onset of macrophage stimulation by cytokines) activate NF-κB and upregulate iNOS (positive feedback). High concentrations have the opposite effect, which may help prevent NO overproduction[36,37]. Both nNOS and eNOS are also transcriptionally regulated by cytokines and other soluble mediators; these effects are generally less striking than with iNOS, however[38].

Enhanced degradation of iNOS protein is one of several mechanisms by which transforming growth factor β (TGF-β) suppresses the production of NO in macrophages, and was the first known instance of post-translational regulation of iNOS[2]. Both iNOS and nNOS are controlled by protein degradation involving the proteasome pathway[39–41]. In macrophages, adding the proteasome inhibitor lactacystin after induction of the iNOS gene by lipopolysaccharide (LPS) drastically increases the amount of steady-state iNOS protein when added[41].

All three NOS isoforms are active only as homodimers. Their dimerization requires binding of calmodulin (which in the case of iNOS occurs at Ca^{2+} concentrations found in resting cells) and incorporation of heme and possibly Zn^{2+} (ref. 3). For nNOS and iNOS, the dimers are further stabilized by binding of tetrahydrobiopterin (BH_4), one of the cofactors of all NOS, and of the substrate L-arginine[3], whose availability is regulated by cytokines (see below). Several proteins block the dimerization and activity of NOS isoforms, including the ubiquitously expressed protein inhibitor of nNOS (PIN), the macrophage product NAP110 (which has 70% amino acid homology to a tumor cell protein that inhibits iNOS) and the central nervous system (CNS) protein kalirin (which also inhibits iNOS and might protect the nervous tissue during inflammatory processes)[42,43].

The eNOS isoform, which is localized as a membrane-anchored protein in the Golgi apparatus and in plasmalemmal vesicles (caveolae) of endothelial and other cells, interacts with several proteins that regulate its activity through positive or negative allosteric effects (for example, heat-shock protein 90 and dynamin-2) or modulation of electron transport (caveolin 1)[44–46]. In one study, a peptide mimicking the caveolin-1 scaffolding domain to which eNOS binds suppressed a carrageenan-induced inflammation in mice as effectively as steroids; this underlines the importance of eNOS for inflammatory responses and of caveolin-1 for the negative control of eNOS[44].

In addition, intracellular redistribution of eNOS can affect NO production. Two products of activated phagocytes, oxidized and hypochlorite-modified low-density lipoproteins, diminish the expression and/or function of eNOS. These were recently shown to impede the production of NO in endothelial cells by reducing eNOS in the plasma membrane[47]. Impairment of endothelial-cell NO synthesis and of NO-dependent vasodilation are thought to be key factors contributing to the development of atherosclerosis.

Another factor that determines NOS activity is the availability of its substrate, arginine. High-output production of NO (for example, by macrophages) depends on extracellular L-arginine even when an adequate level of intracellular arginine is present[48], which argues for the existence of separate arginine pools. In most cell types, uptake of L-arginine occurs via the pH- and Na^+-independent system y^+, whose activity is mediated by a family of cationic amino acid transporter proteins (CAT1, CAT2A, CAT2B, and CAT3) (**Fig. 1a**). In macrophages, CAT1 and CAT2A are upregulated by stimulation with LPS. Macrophages from $CAT2^{-/-}$ mice showed a more than 90% suppression of arginine uptake and NO production after stimulation with interferon (IFN)-γ plus LPS. This indicates that arginine transport *via* CAT2 and iNOS activity form a functional (and perhaps structural) unit[49,50].

Extracellular arginine concentration is strongly modulated by arginase[6] (**Fig. 1a**). This enzyme, which can also be released into the extracellular space, degrades arginine to urea and ornithine and exists in at least two isoforms (cytosolic, 'hepatic' arginase I and mitochondrial, extrahepatic arginase II). In macrophages and bone marrow–derived dendritic cells, T_H2 cytokines (IL-4 with or without IL-10; IL-13), TGF-β, LPS or dexamethasone plus cyclic AMP have been found to strongly increase arginase I[51] or arginase II[52]. The upregulation of arginase prior to the induction of iNOS by IFN-γ plus tumor necrosis factor (TNF) or LPS prevents the NO production by substrate depletion[51–53]. This is independent of a possible inhibition of iNOS gene transcription, protein expression or both by IL-4 and IL-13[17,54]. When both enzymes are coinduced (for example, by LPS), NO production is impaired much less or not at all[48,55], because the K_m value of arginase (I or II) for arginine is approximately 3,000-fold higher than the K_m value of iNOS[3,6].

Macrophages and vascular smooth muscle cells can regenerate arginine from citrulline and thereby utilize citrulline for the production of NO (**Fig. 1a**). Argininosuccinate synthetase, the rate-limiting enzyme of the citrulline–NO cycle, is inducible by LPS (with or without IFN-γ) *in vitro* and *in vivo* in the same cells as iNOS[56–58]. An identical pathway also exists in endothelial cells, in which eNOS, the arginine-regenerating enzymes, and the arginine transporter CAT-1 are thought to colocalize in the caveolae[59].

Cytokines such as IFN-γ, TNF, IL-1, IL-4 and TGF-β induce or suppress guanosine triphosphate cyclohydrolase I, the key enzyme of BH_4 synthesis (**Fig. 1a**). This constitutes another level of post-translational NOS regulation, because BH_4 is essential for NOS catalysis[3,60].

All NOS isoforms can be phosphorylated within cells[3,61]. Although the role of phosphorylation under physiological conditions remains unclear for nNOS and iNOS, serine phosphorylation of eNOS by the Akt kinase is a prerequisite for activity[62].

Regulators of NO production by iNOS

The iNOS isoform is positively or negatively regulated by cell-cell contact (via adhesion and costimulatory molecules), cytokines, immune complexes, microbial and viral products (proteins, lipids, polysaccharides), polyamines, non–ferritin-bound iron, oxygen tension, environmental pH and various antibiotics[2,17,63]. Although IFN-γ and LPS are the prototypic (and still the best-studied) examples, novel regulators continue to be discovered. IL-12 (with IL-18) induces iNOS in various populations of macrophages, through a mechanism mediated by autocrine production of IFN-γ[64]. Among viral and microbial products, the HTLV-I transactivator Tax, the 19-kD lipoprotein of *Mycobacterium tuberculosis* (acting *via* Toll-like receptor (TLR)-2), the flagellin of Gram-negative bacteria (acting *via* TLR-5), the effector protein SopE2 of *Salmonella typhimurium*, bacterial DNA and CpG-containing oligodesoxynucleotides (acting *via* TLR-9) and DNA from various protozoan parasites have all been shown to stimulate NO production by macrophages[65–70]. Regulation of iNOS mediated by cell-cell contact has recently been seen in apoptotic lymphocytes[71]. Uptake of apoptotic (but not necrotic) lymphocytes by macrophages involving the vitronectin receptor and CD36 downregulates the expression of iNOS

and, at the same time, shifts arginine metabolism towards the arginase pathway. This leads to ornithine and putrescine production and to enhanced replication of an intracellular protozoon, *Trypanosoma cruzi*. These effects result from the induction of endogenous TGF-β[71].

NO signaling

The flashing of fireflies on warm summer nights is one of the latest and most impressive examples discovered of the many signaling functions of NO in nature[72]. In the immune system, the use of NO donors and NOS inhibitors and the analysis of $NOS^{-/-}$ mice have provided evidence that NO governs a broad spectrum of processes. These include the differentiation, proliferation and apoptosis of immune cells, the production of cytokines and other soluble mediators, the expression of costimulatory and adhesion molecules, and the synthesis and deposition of extracellular matrix components[9,10,17,73]. Many molecular targets for NO have been identified whose contribution to a specific phenotype remains to be defined (**Table 1**).

Most studies of NO have involved exogenous NO sources (and arbitrarily chosen NO concentrations) and NOS inhibitors with possible side effects, and have been carried out in a wide range of cell types and cell-free systems. It has therefore been impossible to estimate the true extent to which NO exerts positive or negative signaling effects. This problem has recently been tackled by two groups that used high-density oligonucleotide arrays containing 6,500 or 10,703 probe sets (based on cDNAs or expressed sequence tags) to study changes in the gene expression of approximately one-seventh and one-fourth of the mouse genome, respectively[74,75]. The smaller study analyzed the mRNA of hepatocytes from $iNOS^{-/-}$ mice that had been transfected *in vitro* with recombinant adenovirus or a control vector for 24 hours. The authors found that approximately 200 genes (including genes related to inflammation, infection and apoptosis) were subject to regulation by iNOS that led to at least a twofold change in expression level[74]. In the larger study, RNA was prepared from mouse macrophages ($iNOS^{+/+}$ or $iNOS^{-/-}$) that were cultured with or without IFN-γ for 48 hours followed (or not) by infection with *M. tuberculosis*. Using a statistical approach based on reproducibility, iNOS was found to significantly affect the response of 874 genes to IFN-γ, *M. tuberculosis* or both. Similar to the first study, most of these genes were not directly related to immunity and inflammation[75]. Nevertheless, these studies illustrate the considerable influence of iNOS on gene-expression patterns and therefore phenotype.

NO, leukocyte adhesion and chemotaxis

NO inhibits the adhesion of platelets and leukocytes to endothelium. In studies of endothelial-cell monolayers using *in vitro* vascular perfusion systems or flow chambers, both endogenously produced NO and NO donors significantly impede the rolling, firm adherence and/or transmigration of leukocytes (monocytes and granulocytes)[76]. The underlying mechanisms are poorly understood, and no studies have yet been published of the effect of NO on T and B lymphocyte adhesion. NO downregulates the endothelial expression of members

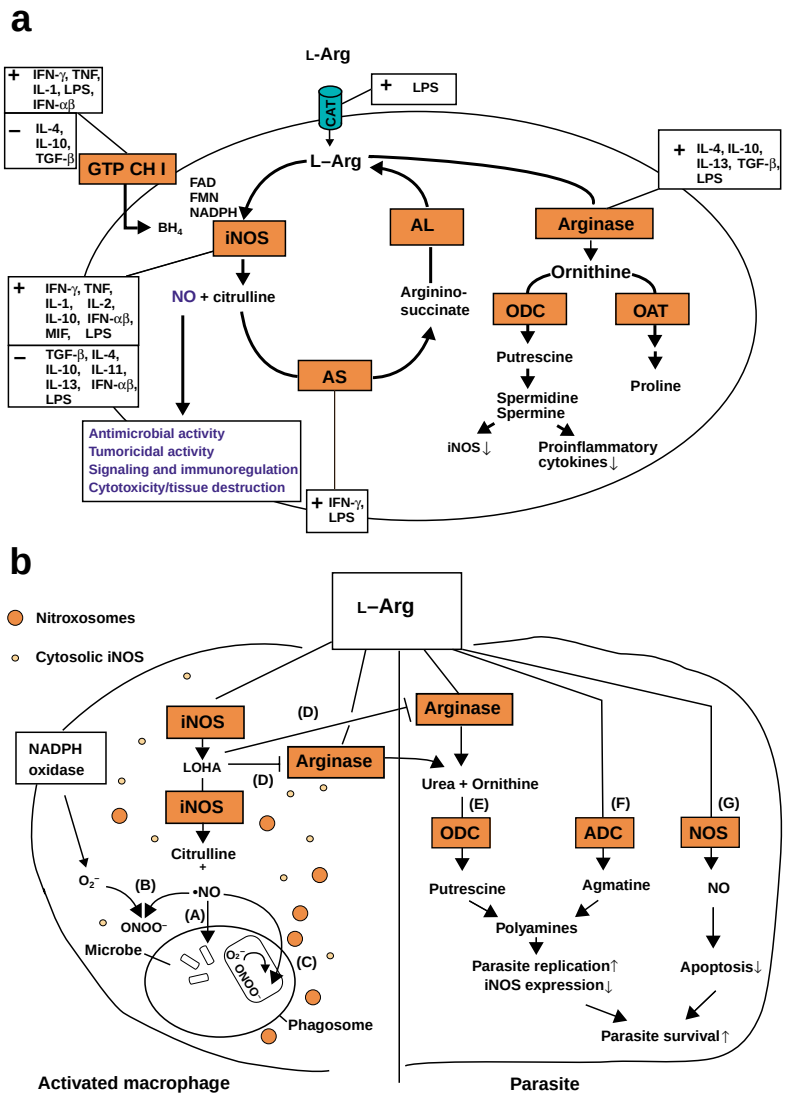

Figure 1. NO pathways and antimicrobial activity. (a) Regulation and function of inducible nitric oxide synthase, arginase and related pathways in mouse macrophages. The activity of iNOS is regulated by cytokines and microbial products (such as LPS), which affect the uptake of L-arginine (L-Arg) by cationic amino acid transporters (CAT), the synthesis of cofactors (such as BH_4 by GTP cyclohydrolase I (GTP-CH I)), the expression of iNOS mRNA and protein, the enzymatic recycling of citrulline to arginine and the depletion of arginine by arginase. Polyamines (putrescine, spermidin, spermin), products of the arginase–ODC pathway, act as immunosuppressants and can further downregulate the production of NO. A high arginase activity in the absence of iNOS can also be associated with tissue fibrosis resulting from the increased synthesis of proline *via* the arginase–OAT pathway[88], which is required for collagen synthesis (for example, by fibroblasts)[6]. AL, argininosuccinate lyase; AS, argininosuccinate synthetase; MIF, macrophage migration inhibitory factor; ODC, ornithine decarboxylase; OAT, ornithine aminotransferase. **(b)** Mechanisms of antimicrobial activity of the L-arginine–iNOS pathway. The antimicrobial activity of iNOS, which is found both in the cytosol as well as an endosomal compartment (nitroxosomes) of macrophages[2], can result from (A) NO radicals or S-nitrosothiols (SNO) or from peroxynitrite ($ONOO^-$) formed by the reaction of •NO with O_2^- generated by the NADPH oxidase of the host cell (B) or produced within the microbe itself (C). On the other hand, iNOS-dependent killing of parasites by macrophages can also be a consequence of the depletion of arginine (D–G). For certain strains of *Leishmania* it was shown that L-hydroxyarginine (LOHA) can inhibit the arginase activity in the macrophage and/or parasite and thereby promote parasite killing (D). Arginine is required for the synthesis of polyamines and DNA in *Leishmania* and African trypanosomes by the ornithine decarboxylase (ODC) pathway (E) and in *T. cruzi* via the arginine decarboxylase (ADC) pathway (F); in *T. cruzi*, which has its own constitutive NOS, it is also used for the synthesis of NO, which acts as an inhibitor of apoptosis and an additional parasite survival factor (G).

of different adhesion molecule families, such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), E-selectin (CD62E) and P-selectin (CD62P), but the extent of modulation was quite variable[77,78]. In addition, NO can inhibit the expression and/or function of integrins on neutrophils, such as CD11a/CD18 (LFA-1)[76,79,80]. In the vasculature of naïve mice, leukocyte rolling and adherence are mainly controlled by NO derived from eNOS and nNOS. During inflammatory responses, leukocyte recruitment and adhesion are also regulated by iNOS[78,80].

NO influences leukocyte chemotactic response by several mechanisms. It can modulate the production of chemokines (such as IP-10, monocyte chemoattractant protein-1 and macrophage inflammatory protein-1α and -2)[17,73,81,82]; inhibit the activity of chemokines (such as IL-8) through peroxynitrite-dependent tyrosine nitration[83] and function as an intracellular messenger in chemokine signaling pathways[84].

NO and the thymus

Because of its capacity to induce apoptosis[15], NO might play a role as effector molecule in the selection and development of T cells in the thymus. In mouse, rat or human thymocytes, iNOS protein is absent[85–87]. By contrast, epithelial and dendritic cells in the corticomedullary junction and medulla of the thymus constitutively express iNOS, which is further upregulated after contact with self antigens or alloantigens or with thymocytes activated by T cell–receptor (TCR) stimulation[85–87]. TCR-activated double-positive thymocytes are highly sensitive to the killing by NO (in particular by peroxynitrite), whereas single-positive thymocytes remain viable upon exposure to NO[85–89]. These data suggest that NO released by iNOS-positive thymic stromal cells is one of the factors mediating deletion of double-positive thymocytes. The function of eNOS expression in thymocytes is still unknown[23].

NO and tumor growth

The inhibition of tumor cell growth and/or induction of tumor cell death by activated macrophages was the first function of NO in the immune system to be discovered[1]. A number of mechanisms have been described whereby macrophage-derived NO can cause cytostasis or kill tumor cells *in vitro* (see **Table 1**). Tumor cell death can also result from iNOS induction within the tumor cells in response to IFN-γ and TNF released by cytotoxic lymphocytes[90]. *In vivo*, $CD4^+$ T cell–dependent production of NO and superoxide by phagocytes (macrophages and eosinophils) is necessary for systemic anti-tumor immunity. Deletion of the iNOS gene and tumor-mediated suppression of macrophage iNOS expression correlate with reduced tumor rejection[91,92]. Production of NO by certain melanoma or sarcoma cells mediated by transfection of the iNOS gene or upregulation of endogenous iNOS prevents tumor metastasis and induces regression of established tumors *in vivo*. On the other hand, iNOS is frequently expressed constitutively in tumor cells. It then promotes tumor growth, neovascularization and invasiveness by induction of p53 mutations and upregulation of vascular endothelial growth factor (reviewed in[17]). Furthermore, exposure of tumor cells to NO leads to an upregulation of the large, catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs), which is required for the repair of double-stranded DNA breaks. The increase in DNA-PKcs protects the cells not only against the toxic effects of NO but also against DNA-damaging agents currently used for tumor therapy (such as x-ray radiation, cisplatin and adriamycin)[93]. All these results must be taken into account when considering NO-based strategies for tumor treatment.

NO and infectious disease

In infectious disease, NO comes into play at all stages and with a diverse spectrum of activities. In the case of vector-borne parasitic diseases, NO can be produced within the vector (protecting it against the parasite), as occurs in *Plasmodium*-carrying *Anopheles* mosquitoes[94]. After reversible binding to salivary proteins (nitrophorins), NO facilitates the vector's blood meal by dilating the blood vessels and antagonizing the hemostatic response of the mammalian host[95]. Tick or sandfly saliva might enhance the initial survival of the transmitted pathogen, as it has been shown to inhibit the production of NO and the killing of *Borrelia* and *Leishmania* by host phagocytes[96,97]. In the infected host organisms, functions of NO described to date include antiviral, antimicrobial, immunostimulatory (proinflammatory), immunosuppressive (anti-inflammatory), cytotoxic (tissue-damaging) and cytoprotective (tissue-preserving) effects. The analysis of $iNOS^{-/-}$ mice unequivocally demonstrates that most of these effects are mediated by iNOS-derived NO[16,17,98,99]. In different cases—depending on the species, strain, infection dose and pathogen entry route—iNOS was indispensable or helped to control the infection, had no discernible effect, or worsened the disease (**Table 3**).

In certain infectious diseases (such as malaria, trypanosomiasis and pneumococcal meningitis) constitutive NOS (especially eNOS) may also have an effect, as suggested by *in vitro* and *in vivo* expression analyses and by phenotypic differences between wild-type mice treated with nonselective NOS inhibitors (inhibiting all NOS isoforms) and of $iNOS^{-/-}$ mice after infection[99–103].

The antimicrobial activity of NO was originally thought to result from mutation of DNA; inhibition of DNA repair and synthesis; inhibitor of protein synthesis; alteration of proteins by S-nitrosylation, ADP-ribosylation or tyrosine nitration; or inactivation of enzymes by disruption of Fe-S clusters, zinc fingers or heme groups or by peroxidation of membrane lipids[14,99]. This conception is still likely to reflect the major proportion of NO's action against infectious agents (**Fig. 1b, A**). One microbicidal molecule might be peroxynitrite ($ONOO^-$), a reaction product of •NO and O_2^-. Peroxynitrite's tyrosine nitrating efficiency and production by macrophages have been a matter of debate because of temporal differences in the activation of NADPH oxidase (the enzyme that generates O_2^-) and iNOS[104,105] (**Fig. 1b, B**). This seems to have been resolved by two recent studies showing that $ONOO^-$ is a potent antibacterial effector molecule and might be formed within the microbes by the reaction of host-derived NO with pathogen-derived O_2^- (**Fig. 1b, C**)[106]. The importance of $ONOO^-$ is underscored by the fact that bacteria such as *M. tuberculosis* and *S. typhimurium* are equipped with peroxiredoxins that detoxify $ONOO^-$ to nitrite[107].

In addition to these direct actions of NO, the antimicrobial activity of the iNOS pathway might also be mediated by indirect effects. Several infectious pathogens (including *T. cruzi*, African trypanosomes, *Giardia lamblia* and *Schistosoma mansoni*) are dependent on exogenous arginine, which they require for the synthesis of polyamines and cell proliferation. Therefore, local arginine depletion by induction of iNOS (or arginase) in macrophages or other host cells can lead to growth inhibition or death of the parasites[108–110] (**Fig. 1b, E–G**). As another possible mechanism of iNOS-dependent control, it was recently suggested that N^{ω}-hydroxy-L-arginine, an intermediate of the L-arginine–iNOS–NO pathway, contributes to the killing of intracellular *Leishmania* in an NO-independent fashion by blocking arginase activity within the parasite and/or the macrophage[111] (**Fig. 1b, D**). This observation contrasts with findings in African trypanosomes demonstrating that arginase inhibition leading to increased arginine availability enhances NO-dependent parasite killing by macrophages[112].

An indirect antimicrobial function of the iNOS pathway is also thought to result from the NO-dependent induction of IFN-γ[113], the NO-

or $ONOO^-$-dependent upregulation of O_2^- and H_2O_2 release by neutrophils[114,115] and the conversion of nitrite into NO_2Cl and $\bullet NO_2$ by myeloperoxidase of neutrophils[7]. Further iNOS-dependent host-protective effects during infectious diseases include the inhibition of tissue fibrosis[98] and the termination of the immune response by apoptosis of activated $CD4^+$ T cells[116]. It remains to be determined whether, during the resolution of infections, iNOS also participates in the regeneration of parenchymal tissues[117], for example by protecting host cells from apoptosis[118] and coordinating the synthesis of extracellular matrix[119].

In several disease models, the antimicrobial and host-protective functions of iNOS/NO are restricted to certain organs and/or stages of the infection. Examples are infections of the liver with *L. donovani*, infections of the liver and spleen with *S. typhimurium*, and aerosol-induced infections of the lungs with *M. tuberculosis*—in each of which iNOS is critical during the late but not the early phase of infection[120–122]; infections with *Toxoplasma gondii*, where iNOS enhances (intestine) or inhibits (CNS) the severity of the disease[99]; and infections with *T. cruzi* (Tulahuen strain), in which iNOS is required for control of the parasites during the acute but not the latent phase of infection[123]. Experiments with $TNF^{-/-}$ mice[124] or $CD4^+$ T cell–depleted mice[125], which succumb to visceral leishmaniasis or tuberculosis despite the expression of high levels of iNOS, clearly demonstrate that additional factors other than iNOS are essential for containing certain pathogens.

In some infections the expression of iNOS is clearly associated with a more severe or even fatal disease outcome. Possible underlying mechanisms include NO-mediated cytotoxicity and tissue damage, inhibition of T cell proliferation and/or induction of T cell apoptosis, generation of viral excape mutants, and direct positive effects on viral or microbial growth[17,21,126,188].

Although most of the results discussed above have been obtained in rodent models, iNOS undoubtedly is also expressed in a broad spectrum of inflammatory diseases in humans[11,12]. The iNOS protein has been detected in alveolar macrophages from patients with pulmonary tuberculosis, in the cerebral cortex of AIDS patients with severe dementia, in peripheral blood mononuclear cells of patients with hepatitis C and malaria, and in the skin of patients with tuberculoid leprosy or localized cutaneous leishmaniasis[17,127]. In patients with leprosy[128] or cutaneous leishmaniasis (M. Qadoumi and C. Bogdan, submitted for publication), reduced tissue expression of iNOS correlates with more severe disease. In patients with *Plasmodium falciparum* infection, death from cerebral malaria correlates with low iNOS expression in the peripheral blood[129,130] and high iNOS expression in the brain[131,132].

NO and transplantation

Several functions of NOS have been seen during the inflammatory reactions that follow allotransplantation. In animal models of cardiac and aortic transplantation, high iNOS expression has been associated with the development of transplant arteriosclerosis. On the other hand, continuous release of NO (derived from iNOS or eNOS) can prevent intimal hyperplasia and protect against the formation of thrombi on the endothelial surface[133]. In rats that have received a renal allograft, inhibition of iNOS reduces tubulointerstitial injury and improves graft function and survival, indicating that iNOS-derived NO contributes to the acute rejection of the organ[134]. Another facet of iNOS is seen in bone marrow–transplanted mice with graft-versus-host reactions (GVHR) directed against major or minor histocompatibility antigens; the GVHR leads to severe immunosuppression (affecting B and T lymphocytes) caused by iNOS-positive macrophages[135].

NO, inflammation and autoimmunity

Table 3. Role of iNOS in infectious diseases (based on results obtained with $iNOS^{-/-}$ mice) (modified from ref.16)[a].

Role of iNOS	Viruses	Bacteria	Protozoa
Dispensable for pathogen control	Mouse hepatitis virus[172] Lymphocytic choriomeningitis virus (liver, spleen, CNS)[170] Sendai virus[188]	*Borrelia burgdorferi*[173] *Chlamydia trachomatis* (vaginal infection) *Helicobacter pylori* *Legionella pneumophila*[174] *Mycobacterium leprae*[175] *Pseudomonas aeruginosa*[174] *Shigella flexneri* *Streptococcus pneumoniae*[103]	*Eimeria vermiformis*[176] *Plasmodium berghei* *Plasmodium chabaudi*[102] *Trypanosoma brucei rhodesiense* (LouTat1)[177]
Essential for pathogen control [b]	Coxsackie virus B3 (myocarditis) Coxsackie virus B3 (pancreatitis)[178] Coxsackie virus B4 Murine cytomegalovirus (intermediate dose)[171] Ectromelia virus	*Mycobacterium tuberculosis* (i.v. infection) *Salmonella typhimurium*[121]	*Leishmania donovani* *Leishmania major* *Trypanosoma cruzi* (Tulahuen strain) *T. cruzi* (Y strain)[179]
Contributory to pathogen control	Hepatitis B virus[180] Lymphocytic choriomeningitis virus (liver)[180] Murine cytomegalovirus (high dose)[171]	*Chlamydia pneumoniae* *C. trachomatis* (spleen, lung) Human granulocytic ehrlichiosis agent[181] *Listeria monocytogenes* (liver, spleen) *L. monocytogenes* (CNS)[182] *M. tuberculosis* (aerosol infection)[122] *Mycoplasma pulmonis* *Staphylococcus aureus*	*Cryptosporidium parvum*[183] *Entamoeba histolytica*[184] *Toxoplasma gondii* (CNS)
Detrimental to the host	Influenza virus	*M. avium* *S. pneumoniae*[103]	*T. gondii* (intestine) *Trypanosoma brucei* (GUTat)[185] *T. cruzi* (Brazil strain)[186]

[a]Owing to space limitations, original references are given only for recent studies that were not discussed in a previous review[99].
[b]iNOS is regarded as essential if any of the following applies: $iNOS^{-/-}$ mice die, control mice survive; non-healing disease in $iNOS^{-/-}$, healing of the disease in $iNOS^{+/+}$ mice; uncontrolled pathogen replication in $iNOS^{-/-}$, pathogen control in $iNOS^{+/+}$ mice.

In autoimmunity, iNOS-derived NO was originally viewed as a tissue-damaging molecule produced by activated macrophages infiltrating the parenchyma[1,13]. Subsequent analyses—mainly in experimental autoimmune arthritis (EAA), encephalomyelitis (EAE), uveitis (EAU) and nephritis (EAN) of rodents—have provided evidence that iNOS also functions as a negative feedback regulator of the autoimmune T_H1 cell response and thereby protects the host against immunopathological sequelae[17,136] (**Table 1**). This view has been complicated by discrepancies between results obtained with iNOS$^{-/-}$ mice and mice treated with NOS inhibitors[136]. For example, in EAA, treatment with L-NMMA (an arginine analogue that inhibits all NOS isoforms) ameliorated the disease, whereas deletion of the iNOS gene (or application of the iNOS inhibitor L-NIL) had no protective effect or even exacerbated the arthritic condition[137,138]. A possible explanation is offered by the findings of McCartney-Francis and colleagues in the streptococcal cell wall–induced arthritis model of rats, in which eNOS and nNOS appear to mediate the acute and chronic erosive joint disease whereas iNOS helped to limit the inflammation[138]. This functional assignment may, however, be premature, because the effects of selective inactivation of eNOS or nNOS have not yet been demonstrated. Nevertheless, the activities of eNOS and nNOS are relevant to the future design of NOS-based therapeutic strategies.

Induction of iNOS also accounts for the prophylactic or therapeutic effect of IL-12 or complete Freund's adjuvant in EAE and EAU, respectively[139,140]. Furthermore, protective anti-inflammatory functions of iNOS have been seen in a T cell–dependent and B cell–mediated myasthenia gravis–like autoimmune disease[141], in local carrageenan-induced pleurisy[142] and in TNF-induced shock of mice[143]. In the latter model, inhibitors of soluble guanylate cyclase (sGC), which is activated by NO, prevented bradycardia, hypotension and lethality normally seen after intravenous injection of TNF. Although the lethal effect of TNF is certainly due partly to NO production, residual iNOS (but not eNOS) activity was strictly required for the rescuing effect of sGC inhibition. Thus, selective inhibition of iNOS is unlikely to protect against TNF-mediated pathologies[143].

Conclusion

In recent years NO has been found to play a much more diverse role in infection and immunity than it was initially assigned. The old ideas that NO is always produced at high levels in the immune system, is derived from iNOS, and has host-protective effects during infection and tissue-damaging effects during autoimmune responses are evidently oversimplifications. It is now clear that iNOS is detrimental in some infectious disease processes and that it helps to counteract excessive immune reactions, protects to some degree against autoimmunity and functions as an intra- and intercellular signaling molecule shaping the immune response. In addition, nNOS and eNOS are now known to participate in important immunological processes such as apoptosis, cell adhesion, autoimmunity and perhaps antimicrobial defense. We have also begun to learn about the possible role of NO in thymic education. The demonstration of iNOS expression by macrophages and other cell types in tissues from patients with a wide variety of infectious, autoimmune and degenerative diseases has disproved the claim that iNOS does not occur in the human immune system. Because the regulation, expression and function of the NOS isoforms are so complex, NO-based therapies against infectious, autoimmune or malignant diseases are not easy to design. This should not, however, discourage immunologists from future research on NO, especially considering that they have been confronted with similar problems in the field of cytokines for years.

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