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***Minireview***

# Nitric Oxide and Immune Response

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Nitric oxide (NO), initially described as a physiological mediator of endothelial cell relaxation plays an important role in hypotension. It is an intercellular messenger and has been recognized as one of the most versatile players in the immune system. Cells of the innate immune system – macrophages, neutrophils and natural killer (NK) cells use pattern recognition receptors to recognize molecular patterns associated with pathogens. Activated macrophages then inhibit pathogen replication by releasing a variety of effector molecules, including NO. In addition to macrophages, a large number of other immune system cells produce and respond to NO. Thus, NO is important as a toxic defense molecule against infectious organisms. It also regulates the functional activity, growth and death of many immune and inflammatory cell types including macrophages, T lymphocytes, antigen-presenting cells, mast cells, neutrophils and NK cells. However, the role of NO in non-specific and specific immunity *in vivo* and in immunologically mediated diseases and inflammation is poorly understood. This review discusses the role of NO in immune response and inflammation and its mechanisms of action in these processes.

**Keywords**: Nitric oxide, iNOS (inducible nitric oxide synthase), Th1/Th2 Cytokines, Immune response, Macrophage, Endothelial cells

## Introduction

The discovery that mammalian cells generate nitric oxide (NO), previously considered merely as an atmospheric pollutant, has provided important information about many biological processes. In 1990s, NO was named the "molecule of the year" and various aspects of its biology have since been reviewed extensively[1]. With a molecular weight of 30, NO is certainly the smallest molecular mediator[2-6]. Furchgott, Ignarro and Murad were awarded Nobel Prize in 1998 for their discoveries concerning NO as a signaling molecule in the cardiovascular system.

Earlier, its role in the immune system was simply defined as a product of macrophages activated by cytokines, microbial compounds or both, derived from the amino acid L-arginine by the enzymatic activity of inducible nitric oxide synthase (iNOS or NOSII) and functions as a tumoricidal and antimicrobial molecule *in vitro* and *in vivo*. But, it has now been recognized that NO plays many other roles in the immune system[7]. First, in addition to macrophages[8,9], a large number of other immune system cells produce and respond to NO. It exhibits wide range of physiologic functions, from immune defense to blood pressure regulation to inhibition of platelet aggregation[7,10]. NO is synthesized from L-arginine by a family of enzymes, the NOS through the L-arginine-NO pathway[11,12]. It has a short life, between 3 and 20s in aqueous and oxygen containing solutions[13] (Fig. 1).

NO plays a crucial role in regulation of vascular tone, neurotransmission, acute and chronic inflammation and host defense mechanisms[14-16]. It is involved in innate immunity as a toxic agent towards infectious organisms, but can induce or regulate death and function of host immune cells, thereby regulating specific immunity[17,18]. It may induce toxic reactions against other tissues of the host and since it is

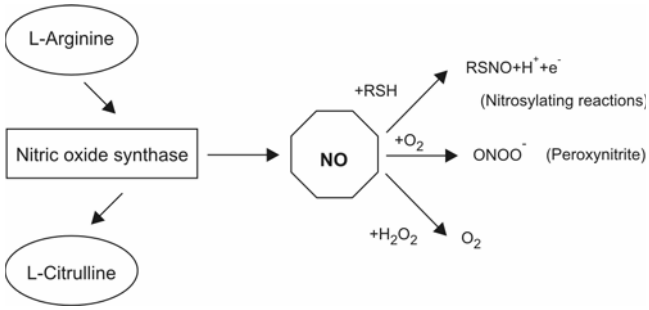


Fig. 1 — Mechanism of synthesis of nitric oxide

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generated at high levels in certain types of inflammation[19,25], for example in asthma[26-28], it has been implicated as a pro-inflammatory agent. It may also act as an anti-inflammatory[29] or immunosuppressive agent[30] via its inhibitory or apoptotic effects on cells[31-33].

The widespread expression of iNOS, following inflammation or infection[34] has been accepted as a vital component of the host's adaptive response to noxious stimuli and virulent pathogens. The increase in NO and its role in the control of leishmaniasis[35,36], malaria[37] and trypanosomal[38,39], viral[40], and fungal infections[41] have been described. Further, peroxynitrite ($ONOO^-$), a potent oxidant formed from NO and superoxide radical ($O_2^-$), has been shown to be microbicidal for various bacteria, including *Salmonella enterica* serovar *Typhimurium*. Nitrosothiols, one-electron oxidized derivatives of NO have been shown to have potent bacteriostatic activity against serovar *Typhimurium*. Pharmacological inhibition of either NO or superoxide production has been shown to result in enhancement of *Salmonella* growth and increased mortality in murine salmonellosis, suggesting that both NO and superoxide contribute critically to host defense against serovar *Typhimurium*. A similar exacerbation of *Salmonella* pathogenesis by *in vivo* blockage of NO biosynthesis has been recently reported.

It has also been demonstrated that mice deficient in both NADPH phagocyte oxidase (phox) and iNOS are more susceptible to various bacterial infections than those either of the two enzymes. Studies using iNOS knockout mice have clearly illustrated the contribution of NO to antimicrobial defense of macrophages against *Salmonella*. Though, inhibition of NO synthesis appears to be a promising treatment in bacterial infections, but early attempts using NOS inhibitors have been largely unsuccessful. However, these have not been selective for the relevant type (iNOS) and simultaneous inhibition of eNOS has been deleterious to organ function[45,46].

Although significance of increase in NO levels in milieu of infection is well recognized, mechanisms by which it aids in host defense remains unclear. Potential mechanism includes direct microbicidal effect via reaction of NO with iron or thiol groups on proteins forming iron-nitrosyl complexes that inactivate enzymes crucial in mitochondrial respiration or DNA replication. In addition, NO has been found to react with superoxide to form reactive oxidants capable of damaging target cells[42-44]. On cellular level, it exerts varied effects on leukocyte cell function, including the induction of macrophage apoptosis, stimulation of macrophage cytoplasmic motility, the modulation of neutrophil adhesion, and the differential regulation of cytokine synthesis[45,46].

There is undoubtedly a close relation between immune response and NO in disease. Thus, it is possible that a reciprocal regulatory mechanism exists between them. This review attempts to briefly describe the mechanisms by which NO selectively controls immune response and how it represents an additional signal for the induction of specific immune response, contributing to the increasingly complex network of immune regulation essential for health and disease.

**Historical perspective of NO**

Two strands of research in the late 1970s led to a revolution in biology. Robert Furchgott found that acetylcholine a neurotransmitter, when injected intravenously lowered blood pressure by relaxing blood vessels, but contracted blood vessels when applied directly in isolation. He showed that the response of blood vessels depended on the innermost layer of cells lining the endothelium. When present, acetylcholine relaxed blood vessels and otherwise contracted them. The relaxing effect of acetylcholine on blood vessels was attributed to the release of a diffusible factor from endothelium termed endothelium-derived relaxing factor (EDRF)[1].

Meanwhile, Ferid Murad found that nitrovasodilators (e.g glyceryl trinitrate, amyl nitrite) used in the treatment of angina, relaxed blood vessels by increasing the levels of cGMP (known to promote relaxation) inside the smooth muscle cells of the vessels, probably by generating NO. Later it was found that EDRF also increased the levels of cGMP in smooth muscle. It was also found that various agents, including haemoglobin and the dye methylene blue inhibited the action both of EDRF and nitrovasodilators. By 1987, Furchgott and Louis Ignarro suggested that EDRF and NO were one and the same. This was confirmed by Salvador Moncada, who showed that NO is synthesized from the dietary L-arginine[11,12].

It then became evident that the endothelium of blood vessels was not the only site where NO is formed. NOS, which uses L-arginine, oxygen and

cofactors to make NO exists in three forms — neuronal NOS (nNOS) from nerve cells, iNOS in inflammatory and other cells when the body's defense mechanisms are activated and *endothelial* NOS (eNOS). nNOS and eNOS are 'constitutive' enzymes in the cells are activated by increase in the intracellular concentration of $Ca^{2+}$ ions. iNOS is produced in response to bacterial infection and other circumstances (such as rheumatoid arthritis) when the immune system is activated and is not regulated by calcium ions[4,10].

**Generation and Regulation of NO in Immune System**

The generation of NO is a feature of genuine immune system cells (dendritic, NK, mast and phagocytic cells including monocytes, macrophages, microglia, Kupffer cells, eosinophils, and neutrophils), as well as other cells involved in immune reactions (such as endothelial, epithelial, vascular smooth muscle, fibroblasts, keratinocytes, chondrocytes, hepatocytes, mesangial and Schwann cells)[12,13,16,47]. The production of NO by macrophages and endothelial cells is quantitatively and qualitatively different from each other. NO production from endothelial cells starts on demand at a low level and is released for short periods in response to receptor activation or mechanical stimulation[48]. In contrast, macrophages are capable of sustained release of high levels of NO initiated by inflammatory cytokines and bacterial products. NO is synthesized universally from L-arginine and molecular oxygen by an enzymatic process that utilizes electrons donated by NADPH. NOS converts L-arginine to NO and L-citrulline via the intermediate *N*-hydroxy-L-arginine. One molecule of L-arginine produces one molecule of NO, the nitrogen atom of the latter deriving from a terminal guanidino group of the arginine side chain. The n and e NOS isoforms renamed NOS I and NOS III, respectively have been fully genetically characterized and are also distributed widely, whereas iNOS or NOS II, originally described in mouse macrophages is expressed in activated cells (Table 1)[49-51].

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. Whether primary T or B lymphocytes express any of the NOS isoforms, is not clear. All NOS isoforms contain flavine adenine diamine, flavin mononucleotide amine and haeme iron as prosthetic groups and require the cofactor tetrahydrobiopterin (BH4). The differential NO production is attributable to isoforms of NOS present in different cells[53,54].

The NOS isoforms appear to be moderately conserved proteins. The NOS I, II and III are encoded by three different genes located on chromosomes 12, 17 and 7, respectively[55,56]. The human genome contains at least two loci for the NOS II gene, one of which (NOS II-1) has been assigned to the proximal region of long arm (cen q11.2 or 11.2-q12) or to pericentric (p11-q11) regions of chromosome 17. Another pseudogene (NOS II-2) is mapped to chromosome 17q11.2 site[57]. The human NOS I gene has 29 exons and extends over 160 kb encoding a protein of approximately 160 kDa (1554 amino acids)[58]. The open reading frame is encoded by 28 exons with translation initiation and termination in exon 2 and exon 29, respectively. The NOS II gene contains 26 exons spanning over 37 kb and encodes a

Table 1 — Characteristics of various isoforms of human NOS[+]

| Properties | NOS I (nNOS) | NOS II (iNOS) | NOS III (eNOS) |
|---|---|---|---|
| Cell source | Neurons | Hepatocytes, neutrophils, airway epithelial cells | Endothelial cells |
| Inhibitors | L-NAME, L-NMMA | L-NAME, L-NMMA, L-NIL, Aminoguanidine | L-NAME, L-NMMA |
| No of amino acids | 1554 | 1153 | 1203 |
| Protein size | 160 kDa | 131 kDa | 144 kDa |
| Gene length | 160 kb | 37 kb | 21 kb |
| No of exons | 29 | 26 | 26 |
| Chromosomal localization | 12 q24.2 | 17 cen-q11.2 or q11.2 – q12 (p11 – q11) | 7 q35-36 |
| Mechanism of activation | $Ca^{2+}$/calmodulin dependent | $Ca^{2+}$/calmodulin independent | $Ca^{2+}$/calmodulin dependent |

[+]Adapted from: Yukio H (1999) Mechanisms of synthesis and action by nitric oxide (NO). *J Clin Expt Med* 191, 417-422

protein of 131 kDa (1153 amino acids)[59,60]. Northern blot analysis shows that human NOS II mRNA is approximately 4.5 kb long. The NOS III gene has 26 exons and it spans over 21 kb encoding a protein of 144 kDa (1203 amino acids)[61]. The deduced amino acid sequences of three human NOS isoforms have shown approximately 50% identity. Across species, amino acid sequences are more than 80% conserved for three isoforms[62,63].

The expression of iNOS is regulated by cytokines[64,65] and determined primarily by the *de novo* synthesis and stability of iNOS mRNA and protein. 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[66,67]. Additional levels of regulation also exist for all three NOS isoforms that may operate during immune responses. Activation of iNOS gene promoter is an important mode of iNOS regulation by cytokines in mouse macrophages, human hepatocyte and epithelial cell lines[3,4,12]. The list of participating transcription factors includes NF-κB, AP-1, the signal transducer and activator of transcription (STAT)-1α, interferon regulatory factor-1 (IRF-1), nuclear factor interleukin-6 (NF-IL-6) and the high-mobility group-I (Y) protein. Depending upon the cytokine, microbial stimulus and cell-type, different upstream signaling pathways are involved in promoting (for eg, 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 inhibiting (for example, phosphoinositide-3-kinase, protein tyrosine phosphatases) iNOS expression. NO itself exerts a biphasic effect on the transcription of iNOS[68]. Its concentrations (such as occur at the onset of macrophage stimulation by cytokines) activate NF-κB and upregulate iNOS (positive feedback), whereas high concentrations have the opposite effect, which may help prevent NO overproduction. Both nNOS and eNOS are also transcriptionally regulated by the cytokines and other soluble mediators, and these effects are generally less striking than with iNOS[68-70].

**Role of NO in Immunity and Inflammation**

NO is synthesized by many cell types involved in immunity and inflammation. The principal enzyme involved is the NOS II, which produces high-level, sustained NO synthesis. NO is important as a toxic defense molecule against infectious organisms. It also regulates the functional activity, growth and death of many immune and inflammatory cell types including macrophages, T lymphocytes, antigen-presenting cells, mast cells, neutrophils and NK cells. However, the role of NO in non-specific and specific immunity *in vivo* and in immunologically mediated diseases and inflammation is poorly understood. NO does not act through a receptor, and its target cell specificity depends on its concentration, chemical reactivity, vicinity of target cells and the way the target cells are programmed to respond. At high concentrations as generated by NOS II, NO is rapidly oxidized to reactive nitrogen oxide species (RNOS) that mediate most of the immunological effects of NOS II-derived NO. RNOS can S-nitrosate thiols to modify key signaling molecules such as kinases and transcription factors. Several key enzymes in mitochondrial respiration are also inhibited by RNOS and this leads to a depletion of ATP and cellular energy. A combination of these interactions may explain the multiple actions of NO in the regulation of immune and inflammatory cells[7-10].

NO-dependent non-specific immunity involves the reticuloendothelial system as well non-reticulo-endothelial cells, such as hepatocytes, vascular smooth muscle, and vascular endothelium, in which iNOS has been detected. The role of the lung and liver in NO-dependent non specific immunity appears to be crucial, since both organs are strategically placed in the circulation to serve as immunologic filters[69,71]. Lymphocytes release NO and murine macrophages reduce lymphocyte activation by a NO-dependent mechanism. These data suggest that NO is also involved in specific immunity, but its precise role is not yet clear. Increasing evidences indicate that NO may play a part in acute and chronic inflammation[7-9]. Treatment with NOS inhibitors reduces the degree of inflammation in rats with acute inflammation or adjuvant arthritis, whereas L-arginine enhances it[48,72,73]. Inhibitors of NOS75 can attenuate immune complex-induced vascular injury in rat lungs and dermal vasculature. Furthermore, colonic synthesis of NO is increased in patients with ulcerative colitis, and NOS inhibitors ameliorate experimentally-induced

chronic ileitis. In addition, nitrite concentrations in plasma and synovial fluid are increased in patients with rheumatoid arthritis and osteoarthritis. The origin of NO in inflammatory process is unclear, but it could probably come from blood vessels, neutrophils and macrophages (Table 2)[76,77].

NO may play a part in tissue damage and may be cytostatic or cytotoxic for invading microorganisms, cells that produce it and neighboring cells. It may also interact with oxygen-derived radicals to generate molecules that could enhance its cytotoxicity. Inhibitors of NOS and NO donors protect against some forms of injury. This is probably due to the dual nature of NO (cytotoxic and vasodilator) and thus is potentially protective[78,79]. NO is, therefore, likely to have a multi-faceted role in inflammatory reactions, ranging from the enhancement of vasodilatation and formation of edema through modulation of sensory nerve endings and leukocyte activity to tissue cytotoxicity[2,4,20,69]. NOS II is expressed in inflammed tissue and a correlation between its expression and disease activity has been observed. NO released from activated macrophages or endothelial cells damage target cells *in vitro* and its high dose can trigger both necrotic and apoptotic pathways of cell death[31,32].

Administration of NOS II inhibitors delays or suppresses autoimmune diseases in animals. However, no amelioration is observed in experimental allergic encephalitis (EAE) and autoimmune diabetes. Furthermore, NO knockout mice show only minor suppression of autoimmune diseases. In some cases, both pharmacological inhibition and genetic inactivation of NOS II even lead to increased disease activity[49]. This may be due to the fact that NOS II is absent during immune cell maturation in knock-out mice[80-83].

Induction of apoptosis (programmed cell death) is important in the regulation of T cell maturation in thymus as well as T cell growth in the periphery[31]. It appears that NO regulates the pathway, leading to apoptosis. At low concentrations, it protects cells from apoptosis by inactivating CPP32-like protease and by increasing Bcl2 protein expression. But, high levels of NO induce thymocyte as well as splenic T cell apoptosis, whereas low doses protect from anti-CD3 induced thymocyte apoptosis. The anti- or pro-apoptotic effects of NO probably involve interaction with simultaneously formed reactive oxygen intermediates and are thus dependent on the redox state of the cell[32,33]. Interestingly, Th1 cells are more susceptible to apoptosis than Th2 cells. It seems that NO regulates the Th1/Th2 balance by promoting or suppressing apoptosis at high/low doses. The cytoprotective properties of low/intermediate levels of NO might limit tissue damage during inflammation, independent of attenuating Th1 responses[84,85,19-22].

NO down-regulates expression of selectins (P and E), VCAM (vascular cell adhesion molecule) and ICAM-1 (intracellular adhesion molecule-1), resulting in suppression of binding to respective ligands on the

Table 2 — Functions of NO in the immune system[+]

| Source of NO (Cell type) | Category | Effector function |
|---|---|---|
| Macrophages, microglia, neutrophils, eosinophils, fibroblasts, endothelial cells, epithelial cells | Antimicrobial activity | Killing or reduced replication of infectious agents (viruses, bacteria, protozoa, fungi and helminths) |
| Macrophages, eosinophils | Anti-tumor activity | Killing or growth inhibition of tumor cells |
| Macrophages, microglia, astroglia, keratinocytes, mesangial cells | Tissue-damaging effect (immunopathology) | Necrosis or fibrosis of the parenchyma |
| Macrophages ('Suppressor phenotype') | Anti-inflammatory-immuno-suppressive effect | Immunoregulatory functions<br>Inhibition of T & B cell proliferation, leukocyte recruitment (adhesion, extravasation, chemotaxis), Antibody production by $CD5^+$ B cells, autoreactive T and B cell diversification |
| Macrophages, T cells, endothelial cells, fibroblasts | Modulation of the production and function of cytokines, chemokines and growth factors | 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 |
| Macrophages | T helper cell deviation | Induction and differentiation of TH1 cells<br>Suppression of TH1 (and TH2) cell responses<br>Suppression of tolerogenic<br>T cell responses |

[+]Adapted from: Yukio H (1999) Mechanisms of synthesis and action by nitric oxide (NO). *J Clin Expt Med* 191, 417-422

vessel wall[86]. Consequently, rolling of leukocytes along the endothelium and migration of cells from vessels to the tissues is also inhibited. Studies suggest that P and E-selectins mediate recruitment of Th1 (but not Th2) cells into inflammed tissues. Since P-selectin expression is down-regulated in the presence of NO, it is clear that NO preferentially down regulates the accumulation of Th1 cells at sites of chronic inflammation by interfering with the adhesion process[86-88].

Higher concentrations of NO inhibit lymphocyte proliferation by Janus kinase[89]. In macrophages, concanavalin A induces NOS II expression and subsequently released NO impairs mitochondrial function and DNA synthesis in T cells, thereby suppressing cell proliferation in certain "low responder" rodents, which can be ameliorated by the addition of NOS II. Recent studies invalidate the non-specific cytostatic effect of NO. Rather, specific impairment of Th1 cell is observed, while Th2 cell function appears unaffected. This is also correlated with the concomitant observation of suppressed IL-2 and IFN-$\gamma$[84,85].

In murine lymphocytes, the IL-2 gene is the target for NO action and exposure to NO suppresses its expression at the level of transcription. Consequently, NO modulates the Th1/Th2 balance by favoring the Th2 response. Exogenous IL-2 can reverse the suppressive effect of NO on Th1 cells. NO induces a similar bias in the human immune system[28]. Here, NO is not found to impair Th1 cell function, but enhances Th2 cell activity by up-regulation of IL-4 production, whereas IL-10 secretion is unaffected. In humans, NO may be limiting Th1 cell activity by down- regulating IL-4 production[90-93].

NO modulates various functions of human phagocytes. In macrophages, it induces transcription of IL-12 p40 gene, but not of human IL-12 p35 gene[90]. Since IL-12 p40 homodimer is an antagonist for IL-12, indicating further less Th1 reactivity in the presence of NO. Similarly, NOS II expression has been reported to contribute to desensitization of macrophages observed after exposure to low concentration of lipopolysaccharide (LPS), and that NO inhibits MHC (major histocompatibility complex) class II expression. NO also inhibits IL-12 synthesis by activated macrophages, thereby indirectly suppressing the expansion of Th1 cells. At low concentrations, it selectively enhances the induction of Th1 cells and has no effect on Th2 cells. It exerts this effect in synergy with IL-12 during Th1 cell differentiation and has no effect on fully committed Th1 cells. NO appears to affect $CD4^+$ T cells directly and not through antigen-presenting cells suggesting an additional pathway by which it can modulate the immune response[6, 84,90-95].

NOS II activity has been found to regulate chemokine production, but it is not clear whether it is another pathway for modulating Th1/Th2 balance[84]. Under certain conditions endogenously produced NO can up-regulate TNF-$\alpha$ production in human phagocytes. The expression of NOS II occurs primarily during Th1 type responses. Hence, NO might serve to limit the extent of potentially dangerous local cellular immune responses. NOS II is also expressed during chronic asthma. It has been suggested that NO supports a Th2 bias of immune reactivity in the lung[96-98].

**Mechanism of Action**

It is not well understood which action of NO is responsible for induction of necrosis or apoptosis. Primary targets for cell death are nuclear and mitochondrial DNA, as well as mitochondrial electron transfer chain and mitochondrial membrane permeability[7]. NO interferes with haeme groups of the electron transfer complex IV. It can also interact directly with DNA, causing deamination. An important additional aspect is uncoupling of the electron transfer chain which gives rise to enhanced production of oxygen free radical[10]. These might react with NO, resulting in the formation of peroxynitrite anion, an extremely potent oxidant. It should be noted that different cell types differ in their resistance to the toxic effects of NO. This might be the result of varying expression of protective molecules, such as hsp70, or different pathways of NO-induced cell death such as necrosis or apoptosis[10-13,31,34].

For the immunoregulatory function of NO, several intracellular targets must be considered. These include the mitochondrial membrane permeability and the nucleus itself. It has been shown that NO disrupts Zn finger configuration by releasing Zn from thiol groups. This leads to reversible inactivation of Zn finger containing transcription factors, and intranuclear Zn release is indeed the result of exposure of live cells to subtoxic NO concentrations. In those cases, where the Zn finger protein antagonizes gene expression, its temporal disruption will allow for transcription to take place. Also, by

reacting with free SH-groups and forming S-NO adducts, other types of transcription factors can be inhibited, including NF-κB. NO also increases expression and prevents degradation of IκB, thereby contributing to further inhibition of NF-κB. Again, both actions of NO tend to attenuate Th1 responses. By contrast, NO mediated transcriptional activation through S-nitrosylation may also occur[45,46,99,100]. In addition to mitochondria and nucleus, additional targets for regulation by NO also exist. In many cases, these are proteins where the functional sites contain Zn-S or Fe-S clusters, or residues, all of which react reversibly with NO. At present, few examples are known in which the inactivation of the proteins outside the mitochondria or the nucleus has a major impact. One such case is the modulation of transcriptional activity, as reported for transferrin receptor and TGF-β[96].

The role of iNOS/NO in the immune system comprises both regulatory and effector functions. The first category includes immunosuppressive effects (e.g inhibition of lymphocyte proliferation) and modulation of cytokine response. The second category includes immunopathologic effects (e.g tissue destruction) and immunoprotective activities (e.g killing of microbial pathogens or apoptosis of autoreactive T cells).

NO is an important mediator of homeostatic processes and host defense. Changes in its generation or actions contribute to pathologic states. The immunoregulatory action of NO primarily targets the Th1/Th2 balance of the immune response[84]. It can induce expression of the Th2 cytokine IL-4, whereas the Th1 cytokine, IFN-γ and IL-2 are suppressed. The apoptosis-inducing activity of NO also affects Th1 cells, as they are more prone to undergo apoptosis than Th2 cells. Interaction of NO with leukocyte adherence might also preferentially affect Th1-cell migration, through inactivation of P-selectin expression[31,85,90]. Taken together, NO released from NOS II might limit the Th1 response at several levels simultaneously. The proposed role of NOS II expression fits in with the observation that mice with a disrupted NOS II gene exhibit enhanced Th1 activity. Any modulation of NOS II expression also affects the Th1/Th2 balance. Recently, several xenobiotic chemicals have been found to modulate NOS II expression. The induction of NOS II by exposure to mercury salts has been found to be responsible for Th2 bias and subsequent pathogenic autoimmune responses.

There is now enough evidence for NO production via NOS II enzyme activity in human tissue during inflammation. Exogenous NO has been shown to induce IL-4 production and shift the Th1/Th2 balance in leukocytes. Smaller amounts of NO might be released in humans and the human cells are more resistant to the cytotoxic actions of NO. Thus, the cytotoxic action of NO towards autologous human immune or tissue cells might be less relevant as compared with its regulatory effect. In humans, the cytotoxic potential of NO is linked to the formation of peroxynitrite, which only occurs at the sites of simultaneous superoxide formation, such as in phagocytes.

The signaling processes through which NO acts to regulate these cells are extremely complex and are only just beginning to be unraveled, but are largely indirect through generation of RNOS that chemically modify enzymes, signaling proteins and transcription factors. Sometimes immune intervention strategies target NOS II as a key mediator for tissue damage in inflammatory diseases[46]. Approaches of this sort must take into account that NOS II also serves to limit destructive Th1 responses. In those cases, where the regulatory role of NOS II exceeds its cytotoxic function, inhibition of NOS II would exacerbate rather than suppress the disease. The role of NO might be different in early or late disease stages[46,47]. For a given cell, the response to NO will depend on its reactivity state, the microenvironment and its tissue type. Therefore, deviation of the Th1/Th2 balance by NO will become apparent at the population level of immune cells rather than at the level of single clones. The two constitutively expressed NOS I and III isoforms, may also be up-regulated to release substantial amounts of NO. However, a contribution of these sources of NO production to immunoregulation in chronic immune responses remains to be elucidated.

## Conclusions

There remain considerable gaps in our knowledge regarding the role of NO *in vivo*, particularly in humans. Future major directions will focus on molecular mechanisms of action of NO, its target molecules and cells and its role in infection and immunologically mediated diseases. Thus, to utilize the capacity of this versatile tiny molecule, we have to

explore a lot to understand its potential yet more thoroughly.

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