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Mini-Review

The regulatory role of nitric oxide in proinflammatory cytokine expression during the induction and resolution of inflammation

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RECEIVED MARCH 16, 2010; REVISED AUGUST 17, 2010; ACCEPTED AUGUST 17, 2010. DOI: 10.1189/jlb.0310149

ABSTRACT

Upon inflammation, neutrophils and subsequently monocytes infiltrate into the involved site. Neutrophils perform functions such as bacterial killing or tissue destruction and then undergo apoptosis, whereas monocytes differentiate into macrophages at the site. Macrophages and other phagocytes finally clear apoptotic neutrophils, leading to resolution of the inflammation. One of the key steps during inflammation is leukocyte infiltration, which is controlled chiefly by chemokines for neutrophils and monocytes. The production of these chemokines is regulated positively or negatively by iNOS-derived NO. Although the mechanisms underlying such dual effects of NO remain unknown, the level of NO and duration of NO exposure appear to be determining factors. The clearance of apoptotic neutrophils without causing further proinflammatory responses, on the other hand, is another key event during inflammation. The production of proinflammatory cytokines appears to be actively suppressed by TGF-β and NO, which are produced by phagocytes upon interaction with apoptotic cells. Overall, NO plays a critical role during inflammation and therefore, remains a potential target for developing therapeutics for inflammatory diseases. ***J. Leukoc. Biol.*** **88: 1157–1162; 2010.**

INTRODUCTION

The hallmark of inflammation is neutrophil and subsequent monocyte infiltration into the involved site. Their infiltration is chiefly governed by various chemokines, such as MIP-2, KC, and MCP-1 in mouse. These chemokines are produced by a variety of cells, including macrophages, neutrophils, endothelial cells, and epithelial cells in response to exogenous stimuli, such as bacteria and fungi, as well as endogenous stimuli, such as damage-associated molecular patterns released from dead cells [1]. The production of chemokines and cytokines is also subject to negative or positive regulation by a variety of products, such as NO and PGs. Consequently, the regulation of neutrophil infiltration has been investigated intensively with the hope of developing therapeutics for inflammatory diseases.

After infiltration, neutrophils then undergo apoptosis, whereas monocytes differentiate into macrophages. The macrophages phagocytose apoptotic neutrophils, contributing to resolution of the inflammation. Therefore, much interest has centered on the mechanism by which the macrophage response is regulated upon clearance of apoptotic neutrophils. Apoptotic cells inhibit the inflammatory responses of phagocytes, such as production of proinflammatory cytokines. Although this has been considered to be an active process mediated by the production of TGF-β, IL-10, PGE_2, and NO [2, 3], its significance in vivo has not been fully elucidated, except perhaps for the case of NO [4].

NO is produced by three different forms of NOS, namely nNOS (or NOS1), eNOS (or NOS3), and iNOS (or NOS2). The former two are constitutive forms and exist as preformed proteins that produce a small amount of NO upon the elevation of intracellular calcium concentration, whereas the third can produce a large amount of NO via de novo synthesis of NOS protein. Because of the huge difference in the amount of NO produced, it has been generally assumed that nNOS and eNOS are critical for a normal physiology, whereas iNOS is associated with injury. In support of this assumption, iNOS-KO mice were less susceptible to DSS-induced intestinal injury than nNOS-KO, eNOS-KO, or WT mice [5].

Compared with nNOS and eNOS, iNOS is expressed in many cell types, including macrophages, neutrophils, DCs, endothelial cells, and epithelial cells [6], raising the possibility that the effect of iNOS-derived NO may depend on the cell type. For instance, iNOS-derived NO plays a physiological role in cardiac myocytes from endotoxemic mice and is essential for the cell-shortening response to β-adrenergic stimulation, whereas iNOS-derived NO plays a detrimental role in neutro-

Abbreviations: DSS=dextran sodium sulfate, eNOS=endothelial NOS, IRAK=IL-1R-associated kinase, KC=keratinocyte-derived chemokine, KO=knockout, nNOS=neuronal NOS, SNAP=S-nitroso-N-acetyl-D, L-penicillamine

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phils and causes a decrease in cell shortening of cardiac myocytes [7].

Whether human macrophages express, produce, or use NO in their host defense functions remains a matter of debate, but readers should refer to a recent review [8]. According to this review, human macrophages also express iNOS in vivo, especially in disease states and chronic inflammatory processes, and it is likely that this NO is functionally relevant to host defense.

In this review, therefore, I will focus on the role of NO in cytokine expression during the induction and resolution of inflammation.

THE ROLE OF NO IN PROINFLAMMATORY CYTOKINE EXPRESSION DURING THE INDUCTION OF INFLAMMATION

The role of NO in inflammation has been studied by using iNOS-KO mice and NOS inhibitors in various types of inflammation models, such as DSS-induced colitis and LPS-induced lung injury. The role of iNOS-derived NO in cytokine expression appears to differ among the models.

As noted in the introduction, DSS-induced injury in the colon was reduced substantially in iNOS-KO mice as compared with WT mice [5], indicating that iNOS-derived NO is harmful in this model. As certain broad-spectrum antibiotics prevent colonic injury [9], it has been assumed that DSS-induced epithelial injury may cause translocation of normal colonic flora and subsequent leukocyte recruitment to the colon. Among leukocytes, neutrophils are effector cells [5], and CXCR2, a receptor for neutrophil chemokines CXCL1/KC, CXCL2/3/MIP-2, and CXCL5/LIX, appears to be crucial in this model [10]. Indeed one of the CXCR2 ligands, KC, was detected in the gut mucosa in this model [11]. To delineate the role of iNOS in bone marrow-derived cells as well as non-bone marrow-derived ones, irradiated WT or iNOS-KO mice were transplanted with bone marrow-derived cells from WT or iNOS-KO mice, respectively [12]. Chimeric-irradiated mice with bone marrow-derived cells of iNOS-KO mice were markedly more resistant to DSS-induced injury, and this resistance was lost when irradiated iNOS-KO mice were given bone marrow-derived cells of WT mice. In this study, neutrophils and epithelial cells were the main source of iNOS in the diseased colon, whereas macrophages exhibited minimal iNOS expression, as evidenced by immunohistochemistry. Therefore, iNOS in neutrophils appears to cause injury in the colon. Moreover, a parallel was found between MPO activity and histology in this study, and MPO activity was lower in the colon of iNOS-KO mice than in that of WT ones, suggesting that iNOS-derived NO enhances neutrophil infiltration. This study found a significant decrease in the MIP-1β and TNF-α mRNA levels in iNOS-KO mice, but it is possible that the levels of neutrophil chemokines such as KC are also reduced in intestinal epithelial cells of iNOS-KO mice, as various NO donors significantly induced IL-8 mRNA in lung epithelial cells via hydroxyl radicals [13]. It should be noted, however, that another study about DSS-induced colitis found that bone marrow-derived, CX3CR1-expressing macrophages were crucial in DSS-induced colitis and that they were a major source of iNOS [14]. The cause for such contradiction is not known but may relate to the different strains used (C57BL/6 in ref. [12] vs. BALB/c in ref. [14]).

Upon airway administration of LPS, an inflammatory response is induced in the lung as a result of activation of phagocytic cells, which produce a variety of chemokines. When inflammatory injury in the lungs was induced by intratracheal instillation of LPS, the injury and MPO activity were increased substantially in iNOS-KO mice [15]. The MIP-2 and KC levels in BAL fluids of iNOS-KO mice, however, were similar to those of WT mice. In contrast, the MCP-1 levels were enhanced in iNOS-KO mice as compared with WT mice. Moreover, administration of anti-MCP-1 antibodies to iNOS-KO mice reduced neutrophil infiltration to the level in WT mice. Consistent with the increase in injury in iNOS-KO mice, in vitro stimulation of microvascular endothelial cells with LPS and IFN-γ elevated the production of MCP-1 in cells from iNOS-KO mice when compared with endothelial cells from WT mice. Peritoneal macrophages from iNOS-KO mice also showed increased production of MCP-1 after stimulation with LPS and IFN-γ. Therefore it is conceivable that when a large amount of NO is produced in the lungs upon intratracheal instillation of LPS, it down-regulates MCP-1 production, neutrophil infiltration into lungs, and lung injury. Although MCP-1 is chemotactic for monocytes, stimulated neutrophils change their chemokine receptor expression pattern and become responsive to certain CC chemokines [16].

In another study about zymosan-induced peritoneal inflammation, neutrophil infiltration at 1 h was enhanced in iNOS-KO mice in comparison with WT mice but suppressed at 2–4 h in KO mice [17]. A notable finding was the parallel between the MIP-2 and KC levels and neutrophil infiltration in zymosan-induced peritoneal inflammation. This suggests that NO exerts a biphasic, regulatory effect on MIP-2 and KC, namely, a suppressive effect at 1 h versus an enhancing effect at 2–4 h. Knowledge of the NO level at each time-point and the producer cell type would facilitate understanding of the biphasic effect of NO.

In addition to the above examples, there have been numerous conflicting papers about the role of NO in the regulation of cytokine expression (**Table 1**). In some cases, NO suppresses the expression of cytokines, such as MCP-1, IL-8, MIP-2, and CCL1 (I-309), whereas in other cases, it augments or induces the expression of cytokines, such as IL-8, MIP-1α, MIP-2, and IFN-β. One explanation for such contradictory effects of NO on cytokine production may be that NO regulates NF-κB in a biphasic manner. In a murine monocyte/macrophage cell line, NO activated NF-κB at early time-points after LPS administration or with lower levels of NO, whereas it inhibited NF-κB activation at later time-points or with higher doses [28]. We obtained similar data for murine resident peritoneal macrophages after coculture with apoptotic cells [3]. When a small amount of NO was produced by macrophages after coculture with late apoptotic cells (secondary necrotic

TABLE 1. Regulation of Cytokine Expression by NO

Cells	Stimulants	Products	Effects of NO donors (+ or –)[a]	Ref.
endothelial cells	LPS + IFN-γ	MCP-1, MIP-2, KC	–[b]	[15]
PM	LPS + IFN-γ	MCP-1[b]	–[b]	[15]
smooth muscle cells	Oxidized LDL or LPS	MCP-1	–	[18]
endothelial cell line	TNF-α	IL-8	–	[19]
HUVEC	glycoxidized LDL	MCP-1	–	[20]
endothelial cells	shear stress	MCP-1	–	[21]
endothelial cells	cytokine	MCP-1	–	[22]
PM	early apoptotic cells	MIP-2	–[c]	[3]
mast cell line	IFN-γ, PMA	IL-8, CCL1	–[c]	[23]
PM	late apoptotic cells	MIP-2	+[c]	[3]
lung epithelial cells	none	IL-8	+	[13]
monocytes	LPS	MIP-1α[d]	+[c]	[24]
lung fibroblasts	hydroxylamine	MIP-2, MCP-1	+	[25]
glomerular mesangial cells	IL-1β	MIP-2	+	[26]
RAW264.7 cells	LPS	IFN-β	+	[27]

PM, Resident peritoneal macrophages; RAW264.7, mouse macrophage cell line. [a]+ Denotes enhancement; – denotes suppression; [b]MIP-2 and KC levels were not changed; [c]Effects of endogenously produced NO; [d]MCP-1 levels were not changed.

cells), the NOS inhibitor, N(G)-nitro-L-arginine methyl ester, and the NO scavenger, 2-(4-carboxyphenyl)-4, 4, 5, 5-tetramethylimidazoline-1-oxyl-3-oxide, each inhibited MIP-2 production, but when a large amount of NO was produced by macrophages after coculture with early apoptotic cells, the NOS inhibitor and the NO scavenger each augmented MIP-2 production through NF-κB activation [3]. The other explanation may be that the effect of NO on cytokine production depends on the type of responder cell, the cytokine, and the stimulant. For instance, NO suppresses MCP-1 but not MIP-2 production by peritoneal macrophages in response to LPS and IFN-γ [18], whereas NO augments IFN-β production by the RAW264.7 macrophage cell line in response to LPS [27].

REGULATION OF NO AND PROINFLAMMATORY CYTOKINE PRODUCTION BY APOPTOTIC CELLS

Apoptotic cells are rapidly cleared by phagocytes, such as macrophages and DCs, without causing inflammatory responses. Likewise, upon inflammation, apoptotic neutrophils are rapidly cleared by macrophages, thereby contributing to resolution of the inflammation. This implies that early apoptotic cells inhibit the inflammatory responses of phagocytes, such as the production of proinflammatory cytokines. This has been considered as an active process mediated by the production of TGF-β, IL-10, and PGE_2, as macrophages cocultured with early apoptotic cells produce these anti-inflammatory mediators that suppress the production of proinflammatory mediators in vitro [2, 29].

The coculture of phagocytes with early apoptotic cells, however, yielded conflicting results concerning NO production and/or iNOS expression in phagocytes (**Table 2**). Unless otherwise stated, early apoptotic cells were used in the studies below.

Henson's group [30] reported that apoptotic cells suppressed the NO production and iNOS induction of macrophages through TGF-β. They stimulated macrophages with LPS and IFN-γ in the presence of apoptotic Jurkat cells and found that as well as producing arginase I, the macrophages produced TGF-β, which in turn, suppressed iNOS and TNF-α induction and NO production. They used a mouse macrophage cell line (RAW264 cells) as well as thioglycollate broth-

TABLE 2. Induction or Suppression of iNOS by Apoptotic Cells

Apoptotic cells/phagocytes	Stimulants	iNOS expression	Ref.
Jurkat/mo TG Mϕ, RAW264	LPS/IFN-γ	suppression	[30]
lymphocytes/mo PM	*Coxiella burnetti*	suppression[a]	[31]
mo splenocytes/mo DC	IFN-γ	induction	[32]
CTLL-2, thymocytes, HL-60/mo PM	none	induction	[3]
thymocytes/mo TM, mo PM	none	induction	[4]
Jurkat/mo epithelial cells (HC-11)	none	induction (mRNA)	[33]
Jurkat/RAW264.7	IFN-γ	no change	[34]

[a]Indirect evidence (see text). mo, Mouse; TG Mϕ, thioglycollate broth macrophage; DC, GM-CSF- and IL-4-induced mouse bone marrow-derived DCs; TM, thymic macrophages obtained after whole body X-irradiation.

elicited macrophages, the latter including inflammatory monocytes and monocyte-derived macrophages. They did not provide data concerning iNOS induction in macrophages by apoptotic cells alone.

C. burnetti, the agent of Q fever in humans and coxiellosis in other mammals, resides in macrophages and induces NO, which then suppresses the formation of large phagolysosome-like replicative vacuoles. Consistent with the findings of Henson's group [30], phagocytosis of apoptotic cells by macrophages reduced the NO production induced by the bacteria, which is dependent on iNOS [31]. As the bacteria induce apoptosis in human monocytes, reduction of NO production by apoptotic cells may represent a counterattack by the bacteria.

Contrary to the results in the above two papers, when myeloid DCs were used as phagocytes, preincubation of DCs with apoptotic cells augmented the response to IFN-γ that leads to the production of NO through iNOS, and NO suppressed the proliferative response of T cells [32]. Although IL-10 was detected in the culture, neither anti-IL-10 antibodies nor anti-TGF-β antibodies restored the proliferative response of T cells. In the absence of IFN-γ, however, apoptotic cells failed to induce NO production in DCs. In view of the findings reported in ref. [32], it is intriguing that IFN-γR-deficient mice were hypersensitive to the anti-CD3-induced cytokine release syndrome and thymocyte apoptosis, in which much less NO was produced [35], as in this case, IFN-γ seems to induce NO production presumably via iNOS, thereby suppressing cytokine production, thymocyte apoptosis, and pathologic manifestations. Therefore, upon induction of cytokine release syndrome by anti-CD3 antibody, it is likely that IFN-γ augments the response of DCs to apoptotic cells, which leads to the production of NO. This possibility may be worthy of further study.

We have shown previously that macrophages cocultured with early apoptotic cells produce neither proinflammatory nor anti-inflammatory cytokines [36] but that such macrophages produce a large quantity of NO to suppress any inflammatory responses such as MIP-2 production [3]. We further showed that upon induction of apoptosis in the thymus by means of X-rays, iNOS-KO mice exhibited higher levels of neutrophil infiltration and production of MIP-2 and KC in the thymus than WT mice [4]. Thymic macrophages isolated from KO mice also produced more MIP-2 and KC than those from WT mice in coculture with apoptotic thymocytes.

Microarray analysis showed that when epithelial cells phagocytosed apoptotic cells, they induced TGF-β2, VEGF-A, and iNOS mRNA in coculture with apoptotic Jurkat cells [33]. Whether the same is true for macrophages, however, has not been reported except for secretion of VEGF.

There has, however, only been one report that exposure of IFN-γ-stimulated RAW264.7 cells to apoptotic cells suppressed NO production but not iNOS expression [34]. Instead of suppression of iNOS, arginase II was increased in these cells, which perhaps reduced the level of arginine. In this case, a lipid factor from apoptotic cells was found to be responsible for modulation of NO production.

Very recently, it was reported that a NO donor, SNAP, activates latent TGF-β1 via activation of soluble guanylate cyclase and generation of cGMP in mouse RAW 264.7 cells [37]. The findings potentially link the suppressive activity of NO with TGF-β.

Although apoptotic human peripheral blood neutrophils have been principally used to study the responses of human macrophages following an encounter with apoptotic cells [38, 39], it should be borne in mind that human peripheral blood neutrophils are at a resting stage, whereas infiltrating neutrophils are activated. In contrast to resting neutrophils, activated neutrophils potentially secrete granular proteins, which may modulate cytokine production [40]. The microenvironment in which apoptotic neutrophils are cleared also warrants attention. Although researchers have often used LPS and/or IFN-γ as stimulants, bacteria may be phagocytosed and killed, and therefore, IFN-γ rather than LPS may be an adequate stimulant for such a study.

MOLECULAR MECHANISM UNDERLYING THE SUPPRESSION OF PROINFLAMMATORY CYTOKINE EXPRESSION BY NO

Various intracellular signaling molecules are regulated by NO, including MAPK, JAK, NF-κB, and AP-1 [41]. These kinases

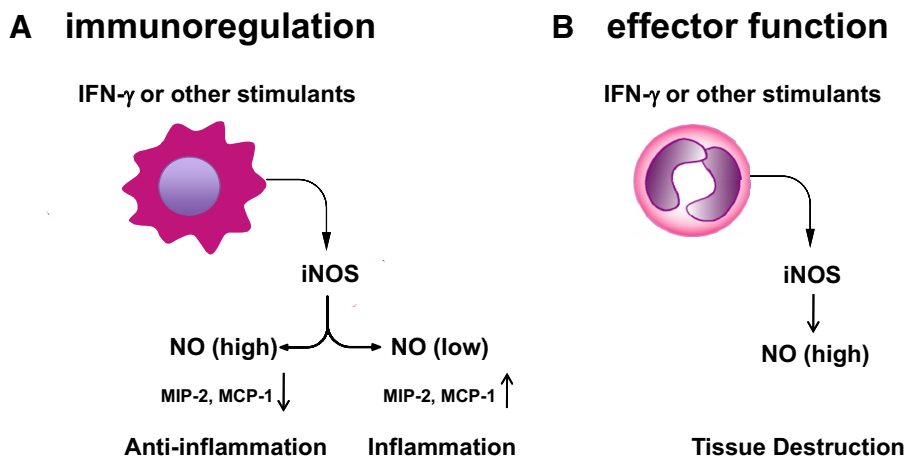

Figure 1. The immunoregulatory effect of NO versus the effector function of NO. (A) When cells such as macrophages are activated with IFN-γ or other stimulants, they produce NO via iNOS. A small amount of NO may enhance the production of chemokines, such as MIP-2 and MCP-1, whereas a large amount of NO may suppresses it. (B) When cells such as neutrophils are activated with IFN-γ or other stimulants, they produce a large amount of NO via iNOS, which has a toxic effect to target cells (effector function).

and transcription factors do not contain transition metals with which NO can interact directly but contain critical cysteine residues that undergo S-nitrosylation and denitrosylation. NO donors, such as SNP and SNAP, directly inhibited the DNA-binding activity of rNF-κB p50 and p65 homodimers and p50-p65 heterodimers. Inhibition of NF-κB p50 DNA binding involved S-nitrosylation of the C62 residue [42]. SNP also caused Tyr nitration of p65 but not p50. Y66 and Y152 nitration of p65 induces its dissociation from p50, its association with IκBα, and subsequent sequestration of p65 in the cytoplasm [43]. The NO donor SNAP also indirectly reduces NF-κB DNA binding. LPS induced IL-12 p40 mRNA expression via IRAK-dependent NF-κB activation, whereas the NO donor inhibited IRAK activation, causing attenuation of its interaction with TRAF-6 and subsequent NF-κB activation [44].

Recent evidence indicates that the HO-1/carbon monoxide pathway is a key player in NO-mediated anti-inflammation [45]. NO reacts with the heme group of soluble guanylyl cyclase, leading to enhanced production of cGMP and HO-1 production and an increase in LPS-induced IL-10 production [46]. It is unknown, however, whether this pathway is involved in NO-mediated suppression of chemokine production during inflammation.

CONCLUDING REMARKS

As iNOS produces a large amount of NO, it has been assumed that such NO is harmful and plays an effector function. This is true of neutrophils (**Fig. 1B**) and perhaps other cells such as macrophages. However, as described in this review, it is evident that NO also plays an immunoregulatory role in the induction and resolution of inflammation. The immunomoregulatory effect of NO appears to be determined by the NO level. A small amount of NO may enhance the production of chemokines, such as MIP-2 and MCP-1, possibly through activation of NF-κB, whereas a large amount of NO may suppress it, possibly through inhibition of NF-κB (Fig. 1A). Therefore, measurement of the NO level and identification of the NO producer cells should facilitate understanding of the effect of NO in vivo.

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KEY WORDS:
iNOS · NO donor · NF-κB