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Nitrosothiols in the Immune System: Signaling and Protection

Pablo Hernansanz-Agustín,[1,*] Alicia Izquierdo-Álvarez,[1,*] Almudena García-Ortiz,[2,*] Sales Ibiza,[3,‡] Juan M. Serrador,[2,†] and Antonio Martínez-Ruiz[1,†]

Abstract

Significance: In the immune system, nitric oxide (NO) has been mainly associated with antibacterial defenses exerted through oxidative, nitrosative, and nitrative stress and signal transduction through cyclic GMP-dependent mechanisms. However, S-nitrosylation is emerging as a post-translational modification (PTM) involved in NO-mediated cell signaling. ***Recent Advances:*** Precise roles for S-nitrosylation in signaling pathways have been described both for innate and adaptive immunity. Denitrosylation may protect macrophages from their own S-nitrosylation, while maintaining nitrosative stress compartmentalized in the phagosomes. Nitrosothiols have also been shown to be beneficial in experimental models of autoimmune diseases, mainly through their role in modulating T-cell differentiation and function. ***Critical Issues:*** Relationship between S-nitrosylation, other thiol redox PTMs, and other NO-signaling pathways has not been always taken into account, particularly in the context of immune responses. Methods for assaying S-nitrosylation in individual proteins and proteomic approaches to study the S-nitrosoproteome are constantly being improved, which helps to move this field forward. ***Future Directions:*** Integrated studies of signaling pathways in the immune system should consider whether S-nitrosylation/denitrosylation processes are among the PTMs influencing the activity of key signaling and adaptor proteins. Studies in pathophysiological scenarios will also be of interest to put these mechanisms into broader contexts. Interventions modulating nitrosothiol levels in autoimmune disease could be investigated with a view to developing new therapies. *Antioxid. Redox Signal*. 18, 288–308.

Introduction

SINCE THE RECOGNITION of nitric oxide (NO) as a second messenger in vertebrate cardiovascular and nervous systems, a classical signaling pathway has been established in which NO produced by nitric oxide synthase (NOS) stimulates soluble guanylate cyclase activation, cyclic GMP (cGMP) production, and cGMP-activated protein kinases (cGKs or PKGs). In addition, a less-classical pathway involves the inhibition of mitochondrial cytochrome *c* oxidase (complex IV of the electron transport chain), which has a profound influence on cell metabolism and homeostasis. Apart from these modes of action, several nonclassical mechanisms have been described, which include NO production from other sources such as nitrite anion (NO_2^-) and the covalent post-translational modification (PTM) of proteins provoked by the so-called reactive nitrogen species (RNS), a series of chemical species derived from reactions of NO with other small molecules [reviewed in Ref. (106)].

Among the PTMs induced by RNS, S-nitrosylation [also called S-nitrosation: see (42, 75, 108) for a discussion of the terminology] has emerged as an important signaling pathway related to NO production, with some particularities in terms of specificity that have been discussed elsewhere (30, 64, 90, 106, 108). It consists in the formation of a nitrosothiol (RSNO, also called thionitrite) at a protein cysteine thiol (RSH), which can potentially be achieved through several possible chemical mechanisms (41, 56, 64). Different structural motifs may confer specificity to particular cysteine residues in protein chains (32, 102), suggesting that these distinct pathways may coexist in any given biochemical environment. As NO itself is rarely a direct S-nitrosylating agent (unless it reacts with a thyil radical in the protein cysteine), most of these

[1]Servicio de Inmunología, Hospital Universitario de La Princesa, Instituto de Investigación Sanitaria Princesa (IP), Madrid, Spain.
[2]Dpto. Biología Celular e Inmunología, Centro de Biología Molecular "Severo Ochoa," CSIC-UAM, Madrid, Spain.
[3]Centro Nacional de Investigaciones Cardiovasculares (CNIC), Madrid, Spain.
*These authors contributed equally to this work amongst themselves.
†These authors contributed equally to this work amongst themselves and are both senior authors.
‡*Current affiliation:* Immunobiology Unit, Instituto de Medicina Molecular, Faculdade de Medicina de Lisboa, Lisboa, Portugal.

mechanisms proceed through formation of RNS. Other important parameters driving the specificity of S-nitrosylation signaling are subcellular compartmentalization and the proximity to or interaction with NOS, as well as the presence of denitrosylases and the recently described transnitrosylase activities (12, 30, 88, 106, 108, 124, 166, 190).

S-nitrosylation is one of several oxidative PTMs produced at cysteine thiols, such as formation of sulfenic, sulfinic, or sulfonic acid, of protein intra- or intermolecular disulfide bridges, or of mixed disulfides with low molecular mass thiols (S-thiolation, termed S-glutathionylation when formed with glutathione [GSH]). The relationships among them are complex, including shared and differential roles in cell signaling and nitroxidative stress (75, 109). In particular, S-nitrosylation has been shown to induce disulfide bridges, and especially S-glutathionylation (5, 109, 183). Indeed, this should be taken into account when studying the effect of NO donors and nitrosothiols, as they can produce different types of modifications (106, 109). For example, S-nitrosoglutathione (GSNO) is not only a nitrosylating agent but also a glutathionylating agent (106, 147, 183).

We review here several recently uncovered aspects of the role of S-nitrosylation in the mammalian immune system. One of them is the influence of S-nitrosylation in toll-like receptor (TLR) activation and signaling, through the modification of several proteins and pathways that are mainly involved in innate immunity, a good example of which is surfactant protein D (SP-D) modification in the alveolar system. Understanding the mechanisms that protect macrophages against their own unbalanced S-nitrosylation when they are activated through pathways that induce high NO and RNS production also reflects the implication of nitrosothiol in innate immunity. NF-kB activation is modulated by S-nitrosylation at many points of its pathway, which is common to innate and adaptive immunity. As such, we discuss this in a middle chapter, together with other proteins and pathways that are S-nitrosylated and that fulfill diverse roles in the immune system. In adaptive immunity, most studies dealing with the role of S-nitrosylation have been conducted on T cells. S-nitrosylation has been described to take part in specific signaling pathways during T-cell activation, while it inhibits T-cell development in the thymus. Finally, we review studies that have described a role for S-nitrosylation and NO production in the differentiation and function of the T-helper cell subsets involved in chronic inflammatory diseases of autoimmune origin, for which treatments with nitrosothiols have been shown to be beneficial in many cases.

In recent years, S-nitrosylation has been shown to play an important role in the plant immune response, sharing given mechanisms with animals [for recent references, see Refs. (100, 196)], even though the existence of NOS has yet to be confirmed in plants (47). While it would certainly be interesting to compare this response in both kingdoms, this is beyond the scope of this review. So is the effect of S-nitrosylation on the microbes that are attacked by the immune system, for example, those engulfed by phagocytic cells, which is covered in another review in this Forum (91a), and in another recent review covering several chemical mechanisms that depend on ROS and RNS (186).

Nitrosothiols in Innate Immunity

An early defense against infectious agents requires the participation of innate immune responses, a set of cellular and biochemical mechanisms representing the organism's first line of defense to pathogens. Physical and chemical barriers (*e.g.,* the respiratory epithelial mucosa and compounds with antimicrobial activity), cytokines, and phagocytic cells (*e.g.,* neutrophils and macrophages) are the main effectors of innate immunity. Among the mechanisms by which phagocytes exert their functions, recent attention has been given to TLRs, a family of transmembrane proteins that recognize microbe-derived molecules, many of which are bacterial wall constituents. TLRs increase phagocytosis and cytokine production mainly through the NF-kB signaling pathway. Ligand binding to the TLR induces the association of the adaptor MyD88 to its cytoplasmic domain, the recruitment of interleukin-1 receptor (IL-1R)-associated kinase (IRAK) and TRAF-6, and the ensuing activation of IκB-kinase (IKK), which in turn activates NF-κB by inhibiting IκB. This enhances the production of the proinflammatory cytokines tumor necrosis factor (TNF)-α, IL-1β, and IL-12 and the expression of adhesion molecules in activated endothelial cells, which favors the recruitment of leukocytes to inflammatory foci.

Role of SP-D S-nitrosylation in TLR activation

Pulmonary surfactant was initially described as a complex of lipids and proteins that reduces the surface tension at the air–liquid interface in the alveoli, thus avoiding atelectasis or collapsed lung. However, recent studies indicate that a surfactant also plays an active role in the innate immune response (89, 157, 188). Four surfactant proteins have been described that belong to the family of collagen-like lectins or collectins: surfactant protein A (SP-A), B (SP-B), C (SP-C), and D (SP-D). The N-terminal domain of the SP-D monomer contains two key Cys residues (Cys15 and Cys20), a collagen-like domain, a neck region formed by a short α-helix, and a C-type carbohydrate-recognition domain (CRD) through which it interacts with anionic phospholipids and complex carbohydrates present in pathogens in a Ca^{2+}-dependent manner (27, 136, 157). It is worth noting that the SP-D monomer does not seem to be biologically active, but rather the monomers interact through their collagen-like domains to form trimers, which can oligomerize into a dodecamer through their N-terminal domains, forming a cruciform structure (27, 136). This new structure is also able to participate in higher orders of multimerization (27).

SP-A and SP-D have a pathogen-dependent proinflammatory function (49), yet conversely, in a noninflammatory scenario, these collectins can bind to the signal inhibitory regulatory protein α (SIRP-α) *via* their CRD, leading to the activation of SHP-1 and inhibition of p38 mitogen-activated protein kinase (MAPK), and thereby suppressing the synthesis of proinflammatory mediators. However, SP-A and SP-D interact with pathogens or apoptotic cells through the CRD, allowing the collagen-like domain at the N-terminus to be presented to calreticulin/CD91, and therefore triggering a proinflammatory response through p38 activation. Thus, SP-A and SP-D can act as both anti-inflammatory and proinflammatory molecules depending on the environment within the lung (49). SP-D hides its N-terminal within the dodecamer structure, so its quaternary structure might be crucial for its immunomodulatory function. Indeed, oligomerization of SP-D (where disulfide bridges form between Cys15 and Cys20) has been demonstrated to be critical for their dual inflammatory role (17, 63, 194).

Recently, S-nitrosylation of Cys15 and Cys20 has been shown to play a pivotal role in oligomerization (7, 57), reviewed in Ref. (6). This modification disassembles the dodecamer into trimers, producing macrophage chemotaxis and triggering a proinflammatory response by activating calreticulin/CD91-dependent p38 (57). Under certain conditions, such as in the presence of pathogen or under nitrosative stress, S-nitrosylation of SP-D could drive the switch between anti- and proinflammatory activity. As mentioned above, dodecameric SP-D would act as an anti-inflammatory molecule in basal conditions. Alternatively, S-nitrosylation of SP-D disassembles the oligomer into trimers, with the N-terminal domain exposed and ready to interact with calreticulin/CD91, promoting the proinflammatory cascade (6, 57) (Fig. 1).

In models of acute lung injury, such as an 8-day exposure to bleomycin, SP-D S-nitrosylation is reduced by 80% in inducible nitric oxide synthase (iNOS) knockout mice, suggesting that this modification is mostly produced by iNOS activity (57). Thus, since iNOS is expressed in inflammatory scenarios, S-nitrosylation of SP-D would produce positive proinflammatory feedback (57).

Some questions still require further study. The oligomeric form of SP-D can bind to TLR4 (132) and inhibit the TLR4-dependent proinflammatory response (194). Thus, it would be of interest to determine whether S-nitrosylation of SP-D might allow TLR4 receptors to dimerize, activating and promoting p38-dependent synthesis of proinflammatory mediators. Likewise, it remains unknown whether S-nitrosylation of Cys15/Cys20 in SP-D is necessary for its interaction with calreticulin/CD91. Finally, it would be very interesting to assess if other oxidative modifications of these Cys residues might have a similar functional relevance.

Macrophage activation and self-protection by denitrosylases

Macrophage activation by proinflammatory cytokines (such as interferon-γ [IFN-γ], produced by other immune cells) or lipopolysaccharide (LPS) (from the bacterial walls) upregulates iNOS transcription, which produces a burst of NO. This NO acts as a part of the antimicrobial armory of these cells, and among other effects (186), it may inactivate through S-nitrosylation essential proteins of phagocytosed cells (146). A murine cell line, RAW 264.7, has frequently been used as a model of macrophage induction to study the role of S-nitrosylation. Early studies showed that when these cells were activated by LPS and IFN-γ, the amount of S-nitrosothiols in cell extracts clearly increases, mainly in the protein fraction (36), which was subsequently confirmed in the same cells (54, 199) and in the J774 macrophage cell line (37). In RAW 264.7 cells, when the intracellular levels of nitrosothiols have been measured after either the treatment of cells with exogenous transnitrosylating agents or the endogenous induction of iNOS, it seems clear that endogenous iNOS induction produces relatively lower amounts of nitrosothiols, even though considerable amounts of iNOS-derived NO are produced (nitrosothiols are estimated at about 0.02% of the nitrite generated) (199, 200). It is worth noting that in J774 cells, bacterial infection was recently reported to reduce the nitrosothiol content of activated macrophages, a process dependent on genes thought to detoxify NO in such bacteria, namely *nor*B

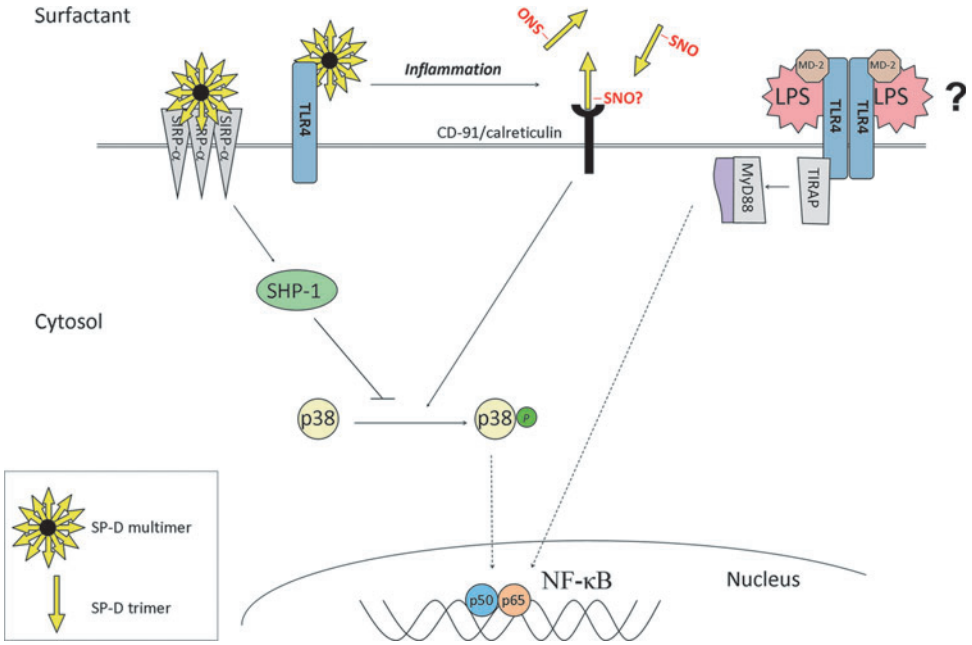

FIG. 1. S-nitrosylation modulates the quaternary structure and function of surfactant protein D (SP-D). The SP-D dodecamer binds to signal inhibitory regulatory protein α (SIRP-α) and inhibits p38 mitogen-activated protein kinase (MAPK) activation *via* SHP-1, and it also binds toll-like receptor 4 (TLR4), thereby avoiding its dimerization and subsequent activation. Upon S-nitrosylation, SP-D dodecamers disassemble into trimers, which bind to CD-91/calreticulin, a process that activates p38 MAPK and triggers a proinflammatory response. Dodecamer disassembly might allow TLR4 dimerization and downstream activation of NF-κB (To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars).

(*Neisseria meningitidis*, meningococcus) and flavohemoglobin, *Hmp* (*Salmonella enterica* and *Escherichia coli*) (92).

Despite producing large amounts of NO, activated macrophages may protect themselves against their own production of such toxic levels of NO and related RNS. It is now clear that denitrosylases may participate in this protection by acting on nitrosothiols in proteins and low-molecular-mass thiols, such as GSH. Two main denitrosylases have been described, which are related to redox pathways: GSH coupled to GSNO reductase (GSNOR) (76, 96); and thioredoxin (Trx), coupled to Trx reductase and NADPH (168) [reviewed in Ref. (12); Fig. 2].

GSNOR (also known as GSH-dependent formaldehyde dehydrogenase) breaks GSNO, and it has been shown to reduce protein S-nitrosylation levels, at least in a subset of proteins whose S-nitrosylation would be in equilibrium with GSH modification (96). Mice with a targeted deletion of GSNOR suffer increased S-nitrosylation and mortality after endotoxic shock induced by LPS, revealing a role in the regulation of innate immunity (98). In a recent report, a GSNOR inhibitor profoundly affected the expression of a number of proteins in activated macrophages (45). By using quantitative general proteomics (*i.e.*, directed at the general proteome and not identifying S-nitrosylated proteins), it has been shown that several inflammatory mediators are downregulated when GSNOR is inhibited, including iNOS, cyclooxygenase-2 (COX-2), and osteopontin, whereas targets of the Nrf2 transcription factor were upregulated, like heme oxygenase-1 and glutamate–cysteine ligase modulatory subunit. These results suggest that a feedback cycle might finely tune the presence and activity of iNOS, in which Nrf2 may also be implicated (45). Another protein susceptible of regulation by denitrosylation is histone deacetylase 2 (HDAC2). HDAC2 is inactivated through S-nitrosylation in alveolar macrophages from chronic obstructive pulmonary disease, which confers cell resistance to corticosteroid treatment. In this setting, GSH treatment and induction of Nrf2 restored HDAC2 activity and corticosteroid sensitivity, although this GSH- and Nrf2-dependent mechanism of denitrosylation was not identified (99).

The role of Trx in denitrosylating particular proteins has been studied in detail for some proteins such as caspase-3 (11, 13) as well as using proteomic approaches (see below). It is not clear if there is an increase in denitrosylation activity during macrophage activation, or if the basal denitrosylation activity is sufficient to cope with the increased RNS produced by iNOS induction. Negative feedback regulators of NOS-dependent S-nitrosylation have been postulated; for example, the Trx-interacting protein (Txnip) that inhibits Trx denitrosylation is in turn inhibited by increased NO levels. Thus, Trx denitrosylation activity is increased when NO is produced (43). In addition, Trx itself is a target of S-nitrosylation at several residues, whose specificity is different in the reduced and oxidized forms (10), and it can act also as a transnitrosylase (118, 189, 190) [reviewed in Ref. (159)]. Therefore, more detailed studies on the different roles of Trx in the regulation of S-nitrosylation in macrophages will be necessary to address this issue.

Further protection could arise through the precise subcellular localization of iNOS in the activated macrophages. Early reports showed that in primary macrophages, iNOS localizes in vesicles that could translocate to phagosomes (178). Each of the three NOS isoforms bears different structural features at the N- and C-termini that influence their subcellular location. Cysteine palmitoylation at the N-terminus of iNOS has been shown to be needed for vesicle association and correct trafficking of the protein through the Golgi apparatus to apical positions in polarized cells, which is needed for the vectorial synthesis and release of NO (125, 126). However, to our knowledge, this has not been studied in macrophages. The four C-terminal amino acids in iNOS, which become inserted

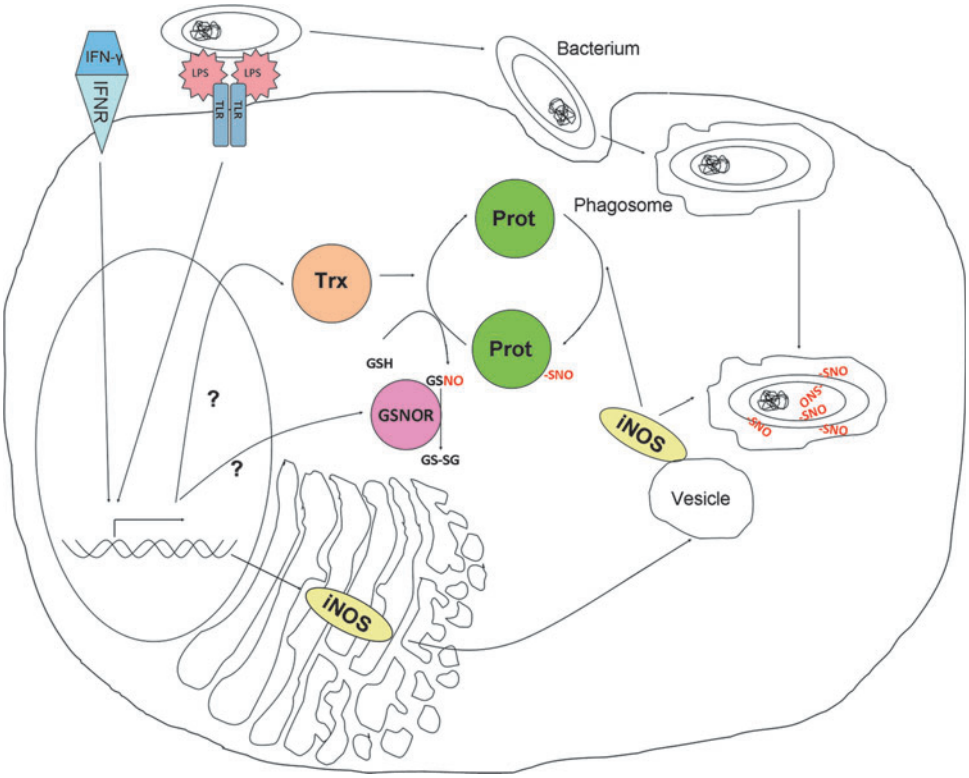

FIG. 2. Macrophages protect themselves from inducible nitric oxide synthase (iNOS)-induced S-nitrosylation. Pro-inflammatory stimuli, such as interferon-γ (IFN-γ) and lipopolysaccharide (LPS), trigger iNOS expression. This iNOS associates with vesicles through the Golgi, and it may be recruited to phagosomes where it produces large amounts of NO that can S-nitrosylate protein targets in a phagocyted cell, such as a bacterium. Thioredoxin (Trx) and glutathione (GSH)/S-nitrosoglutathione reductase (GSNOR) may denitrosylate macrophage proteins that become modified (To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars).

within PDZ domains, are also needed for its apical localization in polarized cells (51, 127). Likewise, in macrophages, the iNOS C-terminus interacts with the PDZ domains of the cytoskeleton scaffolding protein EBP50, which directs iNOS to the phagosomes (29). Interestingly, the *EBP50* gene is induced in parallel to iNOS, and its recruitment is impaired in *Mycobacterium tuberculosis*-infected phagosomes, which explains the previously observed exclusion of iNOS from these phagosomes (29, 116).

Macrophage activation, particularly in the murine cell line RAW 264.7, has been frequently used as a model for the study of the S-nitrosoproteome, illustrating the sensitivity of the proteomic methods employed to identify S-nitrosylated proteins. By 2001, more than 100 S-nitrosylated proteins had been described [see Table S1 in Ref. (167)], all studied on an individual basis. The original biotin-switch technique (BST) described by Jaffrey *et al.* (74) opened the way to study the S-nitrosoproteome, by describing a method that allowed derivatized S-nitrosylated proteins to be purified, and employing emergent proteomic techniques to identify the purified subproteome. However, one study concluded that such methodology only detected S-nitrosylated proteins when RAW 264.7 cells were treated with at least 100 μM extracellular S-nitroso-L-cysteine, which gave an intracellular S-nitrosothiol concentration of around 500 nmol/mg protein (201), well above that produced by endogenous iNOS activation by proinflammatory cytokines (around 100 pmol/mg protein) (36, 54, 199). We obtained similar results, as we were able to detect and identify S-nitrosylated proteins in endothelial cells treated with extracellular S-nitroso-L-cysteine (107, 111), but we did not detect S-nitrosylation differences in RAW 264.7 cells after cytokine-dependent induction and activation of iNOS [results published in Ref. (174), Fig. 3]. However, another study reported an increase in the S-nitrosylated protein signal using the BST, identifying up to 15 proteins modified after cytokine treatment of RAW 264.7 cells (48).

The use of fluorescent derivatization instead of biotin labeling, coupled to two-dimensional electrophoresis (2-DE), has increased the sensitivity of proteomic techniques to study the S-nitrosoproteome. Although the depth of the proteome has not been increased (*i.e.*, the number of identified proteins is not much higher), this approach implies that the starting material may be 20-fold less (174). However, when applied to RAW 264.7 cells activated with LPS/IFN-γ, we were only able to detect differentially S-nitrosylated proteins when we included auranofin, an inhibitor of the Trx pathway, thereby identifying putative targets denitrosylated by this pathway (174). A two-fluorophore scheme for derivatizing S-nitrosothiols has also been used to detect an overall increase in S-nitrosylation in cytokine-stimulated RAW 264.7 cells, mixing samples from nonactivated and activated cells in the same 2-DE (152). Compared to a one-fluorophore one-sample-per-gel scheme (174), this approach has two problems: first, each fluorophore signal has a different normalization parameter; and second, the amount of total protein in each spot from each sample is not analyzed, and thus the variations in the S-nitrosylation signal for a spot may simply be due to a change in protein abundance [reviewed in Ref. (73)].

Development of more sensitive proteomic methods will help to study the S-nitrosoproteome associated with macrophage activation and the role of the protection mechanisms. These advances may come from both general improvements in proteomic techniques, such as the use of more powerful mass spectrometers, as well as through the development of improved protocols and techniques for the specific detection of this modification. Recent advances in the application of quantitative second-generation proteomics (based on large-scale identification of peptides by tandem mass spectrometry), coupled to improved derivatization and purification of S-nitrosylated peptides, could produce advances in the field, even in the detection of S-nitrosylated proteins under basal conditions (32, 44). In the RAW 264.7 cell model of cytokine

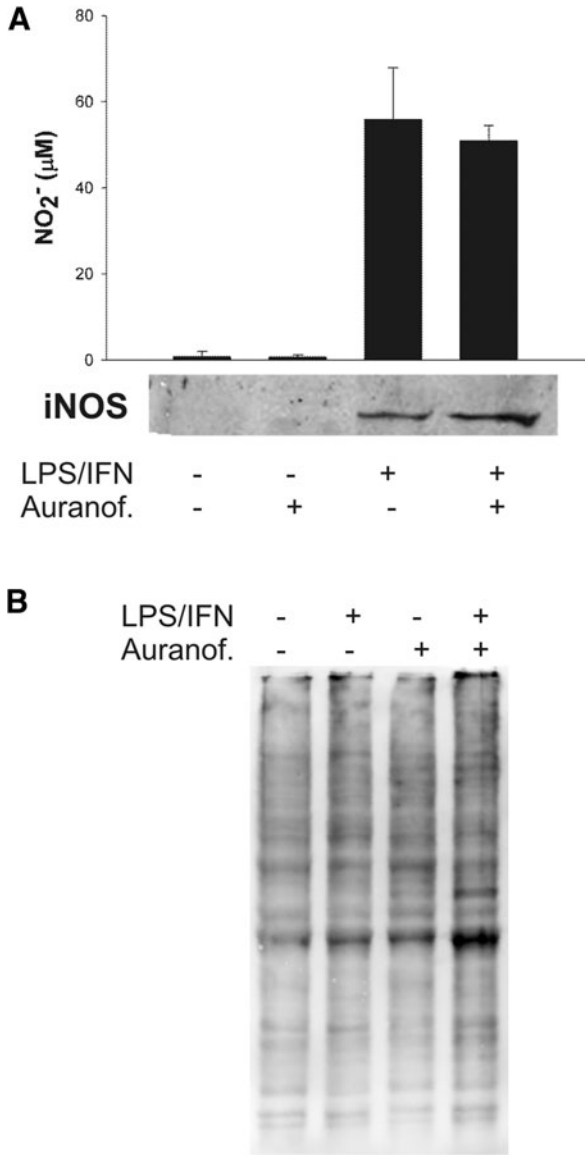

FIG. 3. The biotin-switch technique (BST) is not sensitive enough to detect endogenous S-nitrosylation produced by iNOS activation in macrophages. (A) Murine macrophage cell line RAW 264.7 was treated with LPS and IFN-γ, and with auranofin, producing iNOS that was detected in western blots and NO measured as extracellular nitrite with the Griess reagent. **(B)** Cell extracts were subjected to the BST, blotted, and detected with avidin. Although there is a clear increase in iNOS-derived NO production after LPS + IFN-γ activation, differences in S-nitrosylation are only observed when the Trx pathway is inhibited with auranofin. Reprinted by permission from Tello *et al.* (174).

activation, two methods have recently been applied that use stable isotope labeling in culture or label-free spectral counting, coupled to the purification of derivatized S-nitrosylated peptides. Through these methods, 17 or 27 proteins were described that were S-nitrosylated after cytokine activation (198, 203).

More detailed studies on the S-nitrosoproteome of macrophages during activation, combined with studies into the role of S-nitrosylation in particular proteins, could shed light on the mechanisms that could be operating in self-protection (such as denitrosylation and subcellular localization), as well as on the interaction with phagocytosed cells.

S-Nitrosylation in Innate and Adaptive Immunity

NF-κB pathway and S-nitrosylation

NF-κB is a heterodimeric transcription factor, usually comprised of a p50 and p65 subunit, which controls the expression of many genes involved in innate and adaptive immune responses, including proinflammatory cytokines and adhesion receptors. The activation of NF-κB itself is controlled by a tightly regulated protein cascade. In the basal state, the p50–p65 heterodimer is bound to the inhibitor of NF-κB (IκB), which maintains the transcription factor inactivated. IκB phosphorylation by the IKK complex (IKKα, IKKβ, and IKKγ) promotes IκB degradation by the 26S proteasome and the subsequent liberation, translocation into the nucleus, and DNA binding of NF-κB (50). Distinct stimuli trigger NF-κB activation (*e.g.*, TNF-α, IFN-γ, IL-1β, or LPS) through a series of pathways that converge on the activation of the IKK complex (66).

NO from exogenous sources or endogenous iNOS expression can modulate the promoter and DNA-binding activity of NF-κB in cells. Indeed, some elements of the activation pathway of NF-κB, including the subunits of the transcription factor itself, have been shown to be modified by S-nitrosylation (Fig. 4).

The p50 subunit of NF-κB is S-nitrosylated at a cysteine residue located in its DNA-binding domain, which inhibits its binding to DNA, and thereby decreases the proinflammatory response (104). Interestingly, S-glutathionylation of the same cysteine residue also inactivates its DNA binding (137), a modification likely to be induced by GSNO (86). Likewise, p65 can also be S-nitrosylated, producing similar consequences, and so nitrosylation of both NF-κB subunits results in its inactivation (81). IKKβ has also been shown to be S-nitrosylated at Cys179, a modification capable of modulating kinase function independently of phosphorylation, since IKKβ activity is abrogated after S-nitrosylation after TNF-α activation (145).

Other elements upstream of NF-κB activation are also S-nitrosylated. Treatment of the nonreceptor protein tyrosine kinase $p60^{Src}$ (Src) with nitrosothiol results in Src activation through autophosphorylation at Tyr416 and the formation of multimers, an action presumably exerted through S-nitrosylation at Cys498 and/or the formation of disulfide bridges between Src monomers (3, 5, 142). Furthermore, in the TLR-dependent pathway of NF-κB activation, S-nitrosylation of MyD88 disrupts its binding to the upstream Toll/IL-1R adaptor protein (TIRAP), but not to the downstream IRAK-1, which might influence the delayed development of the acute immune response (70).

CD40 is a member of the TNF receptor (TNFR) family that when stimulated by CD40L activates the NF-κB pathway (20). S-nitrosylation of the CD40 extracellular domain occurs in quiescent macrophages and monocytes, whereas denitrosylation is produced after activation by CD40L (52). This

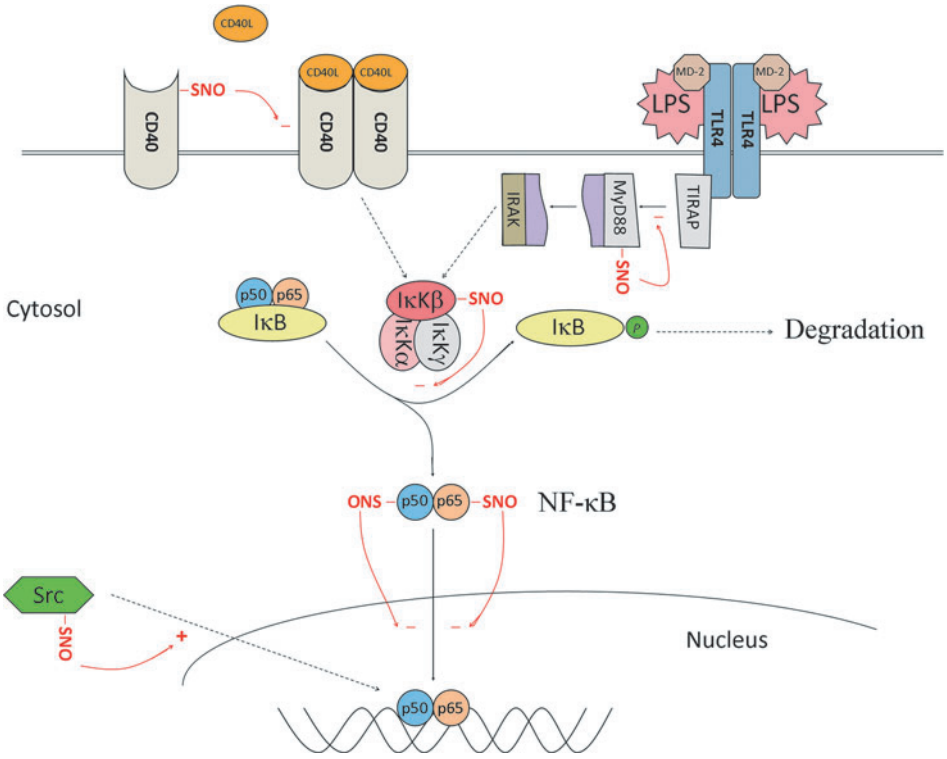

FIG. 4. The NF-κB activation pathway is regulated by S-nitrosylation. S-nitrosylation of the p50 and p65 subunits of NF-κB causes the inhibition of its binding to DNA. Upstream inhibition of the IκB-kinase (IKK) complex's kinase activity is induced by S-nitrosylation of the IKKβ subunit, abrogating NF-κB activation. S-nitrosylation of CD40 inhibits binding and activation by CD40L. MyD88 S-nitrosylation disrupts binding to its upstream partner toll/interleukin-1 receptor adaptor protein (TIRAP), which might serve to delay the development of the immune response. On the other hand, S-nitrosylation of Src may activate NF-κB (To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars).

modification blocks CD40 signal transduction, such that CD40 denitrosylation could be a prerequisite for macrophage activation *via* the CD40 pathway (52). Indeed, CD40 denitrosylation was observed in mice injected with LPS and in monocytes from patients suffering severe sepsis or septic shock (52).

It is worth noting that the *iNOS* gene promoter itself contains NF-κB response elements, and that this transcription factor is important for iNOS expression (81). Thus, it is feasible that negative feedback onto S-nitrosylation-dependent NF-κB inactivation should be taken into account. In this mechanism, inactivation of IKK/NF-κB by S-nitrosylation could downregulate iNOS expression and the associated S-nitrosylation activity, an effect reinforced by the fact that iNOS itself is susceptible to inactivation by S-nitrosylation (117, 163). As a result, NF-κB activation may be equilibrated in cells, and the levels of NF-κB-induced cytokines and endothelial adhesion molecules (vascular cell adhesion protein 1, intercellular adhesion molecule 1, and E- and P-selectins) sustained for leukocyte extravasation and function.

As shown by several groups, S-nitrosylation can affect the NF-κB activation pathway at several points, from the upstream molecules to the transcription factor itself. Although most of the described S-nitrosylation targets (MyD88, CD40, IKKβ, p50, and p65) inhibit the pathway, the modification of SP-D and Src may activate it. Hence, further studies should discriminate how these different modifications are integrated in the regulation of NF-kB.

S-nitrosylation of other relevant proteins and pathways

S100A8 expression is induced by inflammatory mediators of oxidative stress in macrophages, microvascular endothelial cells, fibroblast, and keratinocytes. It has chemotactic-, antimicrobial-, apoptosis-inducing and growth-inhibitory properties (143). S-nitrosylation of S100A8 at Cys41 could be important in the resolution of inflammation given that this modification suppresses mast cell degranulation and mast-cell-mediated inflammation in the microcirculation (95). Interactions between S100A8 and NO produced by endothelial cells may also alter endothelial cell–leukocyte interactions in the microcirculation (95).

C-Jun N-terminal kinase (JNK) belongs to the MAPK family, and it is the terminal kinase in a pathway composed by MEKK1 and MKK4/7. Phosphorylation of JNK allows it to activate target proteins such as c-Jun, a component of the AP-1 transcription factor. This pathway is involved in many different cell activities, including apoptosis, survival, and proliferation. In macrophages activated by IFN-γ, when NO is produced, the activity of JNK is inhibited by a redox mechanism compatible with S-nitrosylation and dependent on Cys116 (134). This effect is independent of its upstream partners and of antagonistic stimuli (134), and it may be mediated by the disruption of the interaction between JNK and c-Jun (135). Interestingly, this cysteine residue is only conserved among the JNK subgroup of MAPK, which suggests a specific role for this mechanism that could participate in resolving the inflammatory response (60). As in the case of the NF-κB pathway, more complex regulation could occur, as c-Jun DNA-binding activity is also inhibited by NO-dependent S-glutathionylation at a critical Cys residue (85), although we do not know of any study specifically assessing S-nitrosylation of that residue.

During the inflammatory process, COX-2, a key enzyme in prostaglandin synthesis, is activated over a similar time course to iNOS, and crosstalk between these enzymes was suspected as iNOS-derived NO had been shown to activate COX (151). In microglia and activated macrophages, iNOS has been shown to bind and S-nitrosylate COX-2, increasing its activity (83). Interestingly, COX-2 S-nitrosylation depends on direct interaction with iNOS, which provides a potential target for the pharmacological control of this pathway (83), stressing the role of S-nitrosylation as a short-range signaling mechanism (106, 110). Indeed, a similar functional interaction with neuronal NOS has been observed in the context of NMDA neurotoxicity (175). Additional synergistic regulation of the COX-2 pathway includes S-nitrosylation of cytosolic phospholipase A2α (cPLA2α), the rate-limiting enzyme upstream of COX, as COX-2 induces the iNOS-dependent cPLA2α S-nitrosylation that activates this enzyme (192).

Adaptive Immunity: Nitrosothiols in T-Cell-Mediated Immune Responses

A role for S-nitrosylation in T-cell activation

In the immune system, helper T lymphocytes orchestrate the responses to pathogenic agents, secreting cytokines whose function is to coordinate the action of leukocytes and immune-associated cells. To fulfill the multiple demands on their effector functions, T lymphocytes proliferate and differentiate in response to the action of T-cell receptor (TCR)-mediated recognition of pathogen-derived antigenic peptides on antigen-presenting cells (APCs) (162). These cognate interactions result in the phosphorylation-dependent activation of signaling pathways initiated from the TCR (112). Besides the importance of protein phosphorylation on key Tyr and Ser/Thr residues in the activation of signal transduction cascades initiated from the TCR, there is increasing evidence indicating that protein S-nitrosylation and its redox switch by denitrosylation are also PTMs able to take part in the regulation of T-cell activation.

T-cell lines and primary T lymphocytes produce NO in response to TCR engagement with CD3 antibodies, superantigens, or antigenic peptides on APCs (69, 165). Despite some controversies regarding the source of NO and the expression of NOS isoforms in immune cells of human origin (155), it has been reported that mouse and human T lymphocytes can express iNOS upon viral infection and in response to proinflammatory cytokines, or other environmental factors (25, 87, 119). Nevertheless, the rapid onset of NO production observed in T cells upon TCR engagement suggests the participation of constitutive rather than inducible NOS. In this regard, human T lymphocytes express endothelial NOS (eNOS), assessed by mRNA and protein detection (122, 144, 156), and although mouse T lymphocytes do not appear to express eNOS mRNA (46), its expression can be induced in murine T lymphocytes upon CD3 stimulation (23). Hence, species differences or conditioning by factors such as stimulation, or the origin and heterogeneity of cell samples, may distort the results of experimental studies.

We reported that eNOS-derived NO is synthesized by T lymphocytes during antigen-specific interactions with APCs (69). eNOS is rapidly activated on the Golgi complex by phosphorylation at Ser1179 due to the combined action of PI3K/AKT signaling and Ca^{2+} fluxes. As a consequence, it is

translocated toward the immune synapse (69), a specialized intercellular domain where the TCR accumulates along with signaling and cytoskeletal molecules, and adhesion receptors, which are organized to regulate the activation of T cells (34). As a result, early and late TCR-mediated signal transduction events are affected, as eNOS-derived NO increases the phosphorylation/activation of the TCR CD3ζ chain and the adaptor kinase ZAP-70. Moreover, eNOS-derived NO also activates the MAPK ERK-1 and ERK-2 through the compartmentalized S-nitrosylation of N-Ras at Cys118 on the Golgi complex, which facilitates its activation by conversion of GDP- to GTP-bound N-Ras (68) (Fig. 5). Moreover, using a specific S-nitrosocysteine antibody, we found that S-nitrosylation is mainly compartmentalized near the Golgi complex, where active eNOS localizes (68), and a similar compartmentalization of S-nitrosylation near the foci of NOS activation has been observed in other cell systems (71). This localization can be explained by the requirement of high NO concentrations to produce S-nitrosylation in comparison with other NO reactions, and it has been postulated as a mechanism for specific S-nitrosylation of proteins interacting or co-localizing with NOS isoforms, suggesting that S-nitrosylation is a short-range or proximity-based NO signaling event (30, 106, 110). We showed that although T cells express both K-Ras and N-Ras (which have the same conserved Cys residue that can be S-nitrosylated), only N-Ras

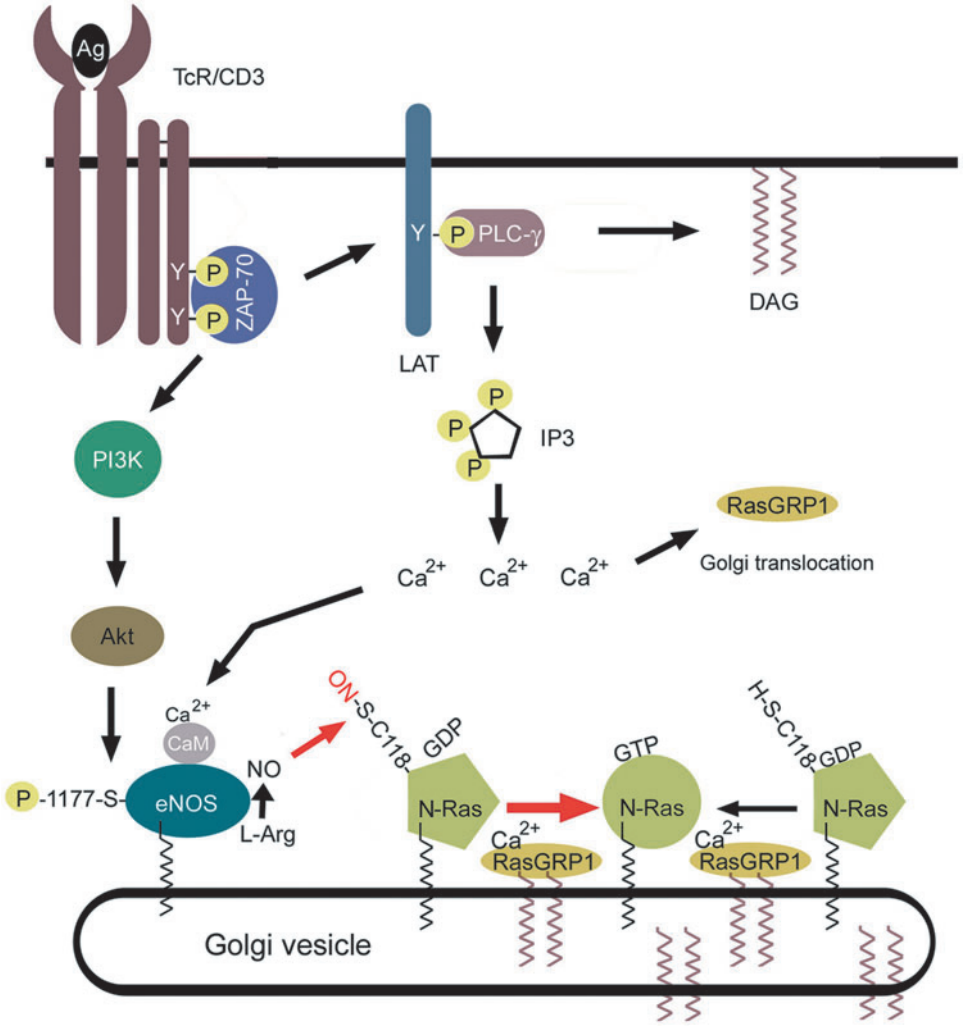

FIG. 5. Model of endothelial nitric oxide synthase (eNOS) activation of N-Ras on the Golgi complex of antigen-stimulated T cells. The figure schematically depicts the possible compartmentalized signaling through which T-cell receptor (TCR) stimulation may lead to the selective S-nitrosylation and activation of N-Ras on the Golgi complex. Engagement of the TCR by antigens results in the phosphorylation of the CD3ζ chain and the recruitment of the adapter kinase ZAP-70. This kinase in turn phosphorylates LAT and PI3K, leading to activation of PLC-γ and Akt, respectively. PLC-γ activation produces diacylglycerol (DAG) in the plasma membrane and inositol 1,4,5-triphosphate (IP3) in the cytosol. IP3 release liberates Ca^{2+} from internal stores, which binds to calmodulin-associated eNOS and induces the translocation of RasGRP1 to the Golgi, where the levels of DAG are high. Simultaneously, active Akt can phosphorylate eNOS on Ser^{1177}. As a result, eNOS might be fully activated to synthesize NO and S-nitrosylate inactive (GDP-bound) N-Ras on Cys^{118} (*small red arrow*). This would weaken its interaction with GDP, thereby facilitating RasGRP1-mediated GDP-GTP exchange on the Golgi complex (*large red arrow*) (To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars).

is S-nitrosylated due to its localization on the Golgi apparatus (68), suggesting that specific targets sensitive to regulation may be S-nitrosylated at the immune synapse.

Our findings also suggest that eNOS-dependent S-nitrosylation of N-Ras on Cys118 may foster activation-induced cell death (AICD), a protective mechanism to avoid the potentially deleterious effects of overactivated T cells (68). Similarly, other authors have found that treatment with GSNO stimulates apoptosis in the human promyelomonocytic cell line THP-1, and that mutation of Ras on Cys118 almost abrogates apoptosis, further suggesting a role of Ras S-nitrosylation in cell death (177). Events leading to apoptosis are activated in the mitochondria through the intrinsic pathway, or in the case of AICD, they are triggered by death receptors such as TNFR and CD95/Fas, a member of the TNFR family that binds to the CD95L/Fas ligand. During AICD, CD95L interacts with the Fas-associated death domain (FADD) and recruits procaspase-8, which is proteolytically activated and released to the cytosol (15).

Although the TNFR may produce antiapoptotic effects through the activation of NF-κB, binding of soluble TNF-α and the formation of dead-inducing signaling complexes associated with the cytoplasmic tail of TNFR may also represent a mechanism to sensitize T cells to AICD. In this regard, nitrosative stress confers to TNF-α the capacity to promote apoptosis in Jurkat T cells by blocking IκBα degradation and the translocation of NF-κB to the nucleus (105). However, rather than inducing apoptosis, low-to-moderate concentrations of NO have been shown to protect T cells from cell death (97).

Caspases are the best-known targets through which NO exerts its antiapoptotic effects. All caspases contain an essential Cys within their active centers that is sensitive to S-nitrosylation, and S-nitrosylation of caspase-1, 3, and 9 is correlated with their enzymatic inhibition in vitro (31, 101). However, studies carried out in Jurkat T cells suggest that rather than inhibiting caspase-3 enzymatic activity, NO attenuates its proteolysis to the active form through both the mitochondrial pathway, by interfering with the Apaf-1/caspase-9 apoptosome assembly, and the CD95 death receptor pathway, by S-nitrosylation of caspase-1 and 8 (31, 197). Another S-nitrosylation-dependent mechanism by which cells may be protected from apoptosis involves Bcl-2. In the human lung epithelial cancer cell line NCI-H460, endogenous NO produced in response to proapoptotic stimuli inhibits Bcl-2 degradation by S-nitrosylation at Cys158 and 229 (9). Nevertheless, in some cell systems, neither caspase nor Bcl-2 S-nitrosylation may be sufficient to fully rescue cells from AICD. In this regard, it has been reported that Ras/MEK/ERK activation promotes intrinsic apoptosis pathways in T cells through the phosphorylation and mitochondrial targeting of the orphan nuclear receptor Nur77. Although in cancer cells Nur77 can bind to Bcl-2 at mitochondria and convert this antiapoptotic factor into a proapoptotic mediator, in T cells, Nur77 exerts its actions through an unknown Bcl-2-independent mechanism (180). Whether eNOS-mediated N-Ras S-nitrosylation on the Golgi complex of T cells may also favor apoptosis through an AICD-independent mechanism, involving ERK-mediated Nur77 phosphorylation/activation, merits further investigation.

There is increasing evidence of a possible role for denitrosylation in T-cell activation. Initial studies on the role of redox maintenance in T cells indicated that GSH is important for proliferation and apoptosis, although it only weakly influences early activation events such as IL-2R expression (114, 153, 164). On the other hand, intracellular GSH levels enable activation of NF-kB in Molt-4 T cells (115), whereas in Jurkat T cells, NO inhibits NF-kB activation through S-nitrosylation of IKKβ at Cys179 (145). Hence, reducing environments maintained by GSH could be required to counteract the inhibitory effects exerted by NO-dependent S-nitrosylation on the proinflammatory transcription factor NF-kB. However, GSH is not the only reducing or denitrosylating agent involved in T-cell activation. Trx-maintained reducing microenvironments facilitate the proliferation of T lymphocytes during antigen-specific interactions with dendritic cells (DCs), and they buffer apoptosis in primary T lymphocytes and Jurkat T cells (4, 72). Trxs are expressed in primary T lymphocytes and T-cell lines, mainly upon mitogenic stimulation in the former (150, 169, 187), and Trx is particularly prominent in T lymphocytes of the intestinal lamina propria. These cells expressed more Trx than peripheral blood T cells (PBTs), and they produce more proinflammatory cytokines in response to activation stimuli (161). Moreover, in experimental studies, cytokine expression and endogenous Trx in activated PBTs increase after pretreatment with recombinant Trx. The importance recently attributed to Trx as a cellular enzymatic system with denitrosylase activity (11, 12, 168) suggests that its expression in T lymphocytes of the lamina propria may be important in intestinal microenvironments as a specialized first line of defense against harmful pathogens, and that this activity may be regulated by S-nitrosylation.

S-nitrosylation/denitrosylation in T-cell development

During the development of immature T cells into $CD4^+$ and $CD8^+$ subsets in the thymus, autoreactive $CD4^+CD8^+$ double-positive thymocytes must be removed by negative selection, where apoptosis is induced when the TCR strongly recognizes MHC-presented self-antigens (160). The mechanisms of apoptosis involved in negative selection in the thymus are not well characterized, although it is known that it differs from that observed in peripheral T lymphocytes, which mainly proceeds through AICD (133). High levels of NO are considered important proapoptotic stimuli that can play decisive roles in T-cell selection in the thymus: TCR-stimulated $CD4^+CD8^+$ thymocytes are highly sensitive to NO-mediated apoptosis, whereas $CD4^+CD8^-$ and $CD4^-CD8^+$ thymocytes are rather resistant (38). This possibility is supported by findings showing that increased NO synthesis by iNOS fosters negative selection of CD3-stimulated double-positive thymocytes (170).

In terms of the mechanisms by which iNOS may regulate negative selection, high levels of NO from S-nitroso-N-acetylpenicillamine have been seen to increase thymocyte apoptosis *via* caspase-1 and p53. Moreover, thymocytes from either caspase-1- or p53-null mice are more resistant to NO-induced apoptosis, supporting the hypothesis that both caspase-1 and p53 may transmit proapoptotic signals induced by NO (53, 202). In fact, it has been proposed that NO increases Bax, but reduces Bcl-2 expression through p53 (53). How the p53/Bax/Bcl-2 axis regulates caspase-1 activation in thymocytes is a subject of active research. Strikingly, recent studies indicate that S-nitrosylation/denitrosylation is required for T-cell development. In the thymus of GSNOR-

deficient mice, there is increased S-nitrosylation (with GAPDH one of the major proteins identified), apoptosis, and concomitant reduction of $CD4^+$ thymocytes, a situation that was normalized in the thymus from iNOS and GSNOR double knockout mice (195). DCs of the corticomedullary junction and medulla constitutively express iNOS in the thymus, and this expression is upregulated after their interaction with thymocytes activated with auto- or alloantigens (2, 170). Hence, NO from thymic stromal DCs may be an important secondary signal associated to TCR-mediated apoptosis, and its effects on negative selection may take place through S-nitrosylation, which can be counteracted by the denitrosylase activity of GSNOR (Fig. 6). Interestingly, recent studies carried out in macrophages and neuroblastoma cells indicate that iNOS-derived NO S-nitrosylates GAPDH, and that once S-nitrosylated, GAPDH can translocate to the nucleus after interacting with the E3-ubiquitin ligase Siah1, where it activates the acetyl transferase activity of p300/CBP and induces apoptosis by increasing p53 expression (62, 158). Further work will be required to find out whether S-nitrosylation/denitrosylation exchange on GAPDH actually regulates p53-mediated apoptosis during T-cell development.

By contrast, there is evidence that the selection of $CD8^+$ and $CD4^+$ T lymphocytes is no different in iNOS-deficient and wild-type animals (173), suggesting that the denitrosylase activity of GSNOR during T-cell development may predominate over the S-nitrosylation activity of iNOS. It remains unclear how $CD4^+$ maturation is selectively regulated by GSNOR. One possibility is that thymocytes ongoing $CD4^+$ maturation may be more sensitive to S-nitrosylation-mediated apoptosis than $CD8^+$ thymocytes. If so, MHC class II-restricted signals from DCs may upregulate GSNOR expression/activation in thymocytes undergoing $CD4^+$ maturation, preferentially counteracting the proapoptotic actions of iNOS-derived S-nitrosylation in those cells. Furthermore, iNOS-independent S-nitrosylation may also be important for T-cell development. In this regard, it has been reported that mouse

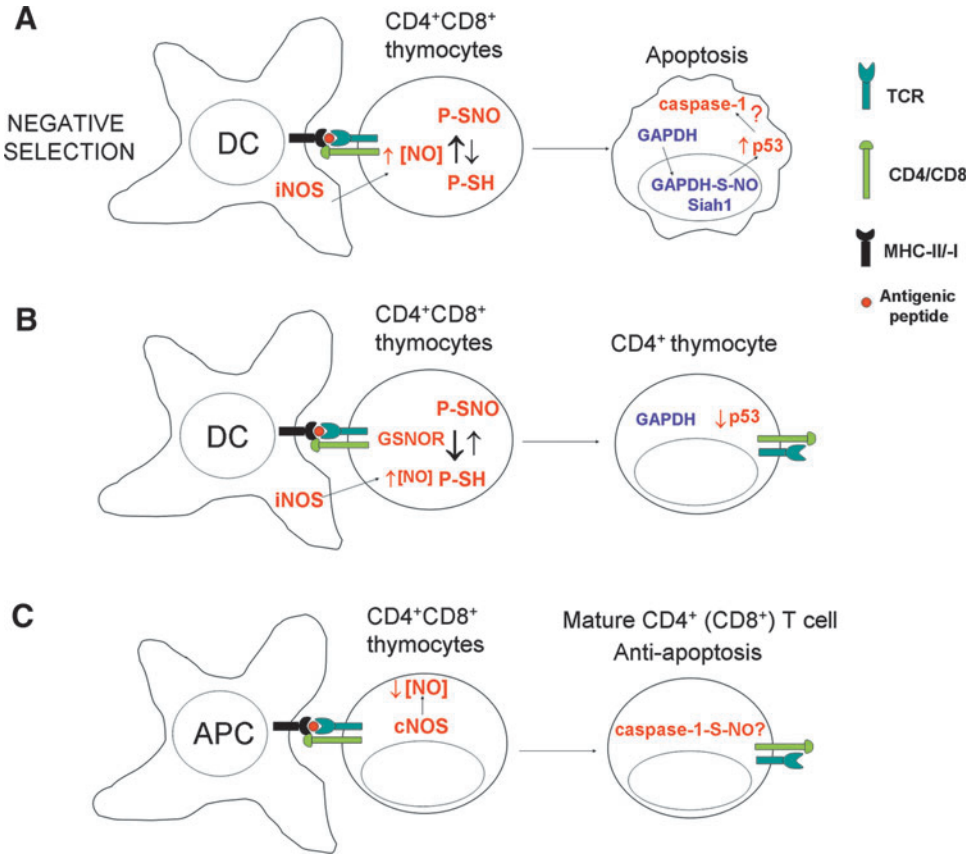

FIG. 6. Hypothetical S-nitrosylation/denitrosylation mechanisms in T-cell development. (A) Local high extracellular levels of NO produced by iNOS from corticomedullar and medullar dendritic cells (DCs) in the thymus promote S-nitrosylation in thymocytes, fostering negative selection of $CD4^+CD8^+$ double-positive thymocytes. Protein S-nitrosylation in thymocytes may induce apoptosis by increasing p53 expression. The iNOS-derived NO may promote apoptosis through the S-nitrosylation of GAPDH. S-nitrosylated GAPDH can bind to Siah1 and translocate to the nucleus, where it increases the acetyltransferase activity of p300/CBP and the ensuing synthesis of p53, which can in turn activate caspase-1 through a still-to-be determined mechanism. **(B)** Through denitrosylation, upregulated GSNOR expression or activity in thymocytes ongoing $CD4^+$ maturation may counteract the proapoptotic actions of protein S-nitrosylation mediated by iNOS-derived NO from DCs. **(C)** Low levels of NO generated in thymocytes by constitutive NOS (cNOS) during their cognate interactions with iNOS-nonexpressing DCs and macrophages (antigen-presenting cells [APC]) may protect thymocytes from cell death by S-nitrosylation of caspase-1 (To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars).

thymocytes can express constitutive NOS as well as iNOS, and produce low levels of Ca^{2+}-induced NO, even in the presence of iNOS-inducing stimuli such as LPS and proinflammatory cytokines (19, 28, 185). It is yet to be seen whether the antiapoptotic actions of caspase S-nitrosylation generated by NO from constitutive NOS might also play an important role in modulating apoptosis during thymocyte maturation.

NO and nitrosothiols in T-cell differentiation and autoimmunity

Naive $CD4^+$ T lymphocytes recognize antigens in peripheral lymphoid organs, provoking the expansion and differentiation of antigen-specific lymphocytes into subsets of effector cells that can be distinguished on the basis of the cytokines they produce. Chronic inflammatory diseases are often dominated by Th1, Th2, and Th17 cells and modulated by regulatory T cells (Tregs) (94). The most important differentiation-inducing stimuli for Th1, Th2, and Th17 cells are IL-12, IL-4, and IL-6/IL-23, respectively. The main function of Th1 and Th17 cells is to activate cellular immunity: Th1 cells produce IFN-γ and TNF-α, whereas Th17 cells produce IL-17 and IL-22. On the other hand, Th2 cells regulate humoral immune responses typically synthesizing IL-4, IL-5, IL-6, and IL-10, whereas Tregs mainly produce IL-10 and have been implicated in the generation of tolerance to exacerbated inflammatory responses (94).

The regulation of cytokines by NO has received considerable attention, because it might be relevant in the selection of adaptive immune responses and the management of autoimmune chronic inflammation (Table 1). The first solid evidence of a role for NO in T-cell differentiation came from a seminal study carried out in iNOS-deficient Balb/c mice, which generated Th1 responses upon *Leishmania major* infection instead of the characteristic Th2 polarization associated with control Balb/c mice (182). Later studies demonstrated that high NO concentrations promoted Th2 differentiation by suppressing IL-12 synthesis in activated macrophages (67). Since then, iNOS has been associated with the incidence and severity of many chronic inflammatory diseases, such as rheumatoid arthritis (113, 121), systemic lupus erythematosus (123), experimental autoimmune uveitis (EAU) (172), experimental autoimmune encephalomyelitis (EAE) (184), and asthma (58).

Th1 Differentiation. Several reports support the hypothesis that nitrosothiols may be behind the actions of NO in Th1 differentiation. In a murine model of autoimmune hepatitis induced by concanavalin-A activation of Fas-regulated apoptosis (148, 176), high levels of NO from NO-aspirin (NCX-4016) or from a NO derivative of ursodeoxycholic acid (NCX-1000) reduced Th1 responses by inhibiting the caspases involved in the processing and production of Th1 cytokines (39, 40). This effect was reverted when cell lysates were treated with DTT or $HgCl_2$, which are able to reduce oxidized cysteines (including S-nitrosothiols), suggesting that the inhibitory effect of NO on caspase activity could be achieved *via* the S-nitrosylation of essential cysteine residues within their catalytic site (39).

Moreover, Trx induction in T lymphocytes increased production of Th1 cytokines by stimulating *IFN-γ* gene expression (79). Therefore, it seems likely that high levels of NO generated by iNOS may prevent overexpansion of Th1 cells by S-nitrosylation of key transcriptional and post-transcriptional regulators of Th1 pathways. By contrast, low concentrations of NO stimulate T cells to express IL12-Rβ2 and promote Th1 differentiation *via* cGMP (130). Nevertheless, since Th1 cells are more sensitive to apoptosis induced by high concentrations of NO than Th2 cells (149), and low-to-moderate concentrations of NO can exert S-nitrosylation-mediated antiapoptotic effects, the influence of NO from constitutive NOS may improve Th1 viability and influence the Th1/Th2 balance. Indeed, it has been reported that Ras/MAPK signaling also plays an important role in T-cell differentiation: strong signals from the antigen-engaged TCR sustain ERK activation and favor Th1 differentiation, whereas weak and transient TCR-mediated activation of ERKs preferentially induces Th2 differentiation, a consequence of increased Jak-1, STAT-6, and GATA-3 activation (193). A possible role for NO in Ras/ERK-mediated Th1/Th2 differentiation is supported by eNOS and NO donors regulating ERK activity through Ras S-nitrosylation (68, 91). Nevertheless, a recent study analyzing eNOS-deficient C57BL/6 mice infected with *L. major* clearly showed that eNOS does not play any role in the selection of the Th1/Th2 immune response to infection, although it enhanced granulocyte infiltration into lesions (46). However, it remains possible that the actions of eNOS-derived NO in human and mouse T-cell differentiation could be species specific (see above).

Uveitis represents a group of sight-threatening intraocular inflammatory diseases, including Behçet's disease, birdshot retinochoroidopathy, sympathetic ophthalmia, Vogt-Koyanagi-Harada's disease, and ocular sarcoidosis, which may have an autoimmune etiology (131). EAU can be induced in susceptible rodent species by immunization with the retinal protein, interphotoreceptor retinoid-binding protein (IRBP) (181). GSNO treatment ameliorates the disease due to its ability to inhibit retinal expression of Th1 cytokines and T-cell proliferation (61). In an earlier report, administration of IL-12 to these animals protected them from disease due to apoptotic deletion of detrimental T cells, inducing IFN-γ overproduction and iNOS-derived NO (172). While not specifically assessed, these results suggest that Th1 cells could be the subset of detrimental T cells depleted. More recent work indicates that uveitis is also associated with Th17 response (24, 181), although we have not found reports of nitrosothiols participation in Th17-dependent uveitis.

Th17 Differentiation. Multiple sclerosis (MS) is a common inflammatory disease of the central nervous system (CNS) characterized by the instability and disruption of myelin, the death of oligodendrocytes and axonal damage (80). A murine EAE model of MS involves the induction of a neuroinflammatory disease by immunization with antigens derived from myelin protein. This animal model is associated with impaired integrity of the blood–brain barrier, upregulation of adhesion molecules, and the invasion of vascular inflammatory cells into the CNS.

In the recent years, increasing importance has been given to Th17 cells as mediators of EAE, and NO has emerged as a possible regulator of Th17 proliferation and function. NO inhibits the proliferation and function of polarized Th17 cells, and EAE is more severe in iNOS-deficient mice than in wild-

Table 1. Role of S-Nitrosylation in Autoimmune Diseases

Disease (model)	*T-cell response*	*Cytokines implicated*	*Endogenous NO source*	*Exogenous treatments*	*Implications for disease*	*Mechanism of action of nitrosothiol/NO*	*References*
Uveitis (EAU)	Th1* Th17*	IFN-γ TNF-α IL-10 IL-17	iNOS	GSNO	Disease improvement	Inhibition of proinflammatory cytokines and T-cell proliferation	(61, 172)
Multiple sclerosis (EAE)	Th17	IL-17 TNF-β IFN-γ IL-1β	iNOS eNOS	GSNO	Disease improvement	NO inhibits proliferation and function of polarized Th17 cells GSNO protects myelin GSNO produces differences in the S-nitrosoproteome	(139) (128) (14, 191)
Asthma	Th2		iNOS	SNAP	Disease improvement	NO suppresses proliferation of activated Th2 cells S-nitrosylated proteins reduced in asthmatic patients	(35) (140)
Autoimmune hepatitis	Th1	Th1 cytokines		NO donors (NO-aspirin, NCX-1000)	Disease improvement	Probable S-nitrosylation of caspases catalytic Cys, inhibiting Th1 response	(39, 40)
Rheumatoid arthritis		MBL			Harmful effects on disease	S-nitrosylation of MBL	(59, 65)
Crohn's disease				GSNO	Disease improvement	Reduced intestinal permeability Increases ZO-1 mRNA and protein levels	(154)

*There are not enough evidences for uveitis to be caused by a Th1 or Th17 cell response.

EAU, experimental autoimmune uveitis; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; GSNO, S-nitrosoglutathione; IFN-γ, interferon-γ; SNAP, S-nitroso-N-acetylpenicillamine; MBL, mannose-binding lectin; TNF, tumor necrosis factor; IL, interleukin.

type controls (128). Moreover, GSNO attenuates EAE, protecting myelin by restricting mononuclear cell infiltration and inhibiting proinflammatory mediator expression (TNF-β, IFN-γ, and IL-1β), as well as by reducing IL-17 production through the inactivation of STAT-3 and the reduction of RORγ expression, two essential transcription factors for Th17 cell differentiation and function (139).

In EAE mice, differences in the S-nitrosoproteome have been described by comparative biotin-switch and western blot analysis, affecting cytoskeletal components (neurofilament proteins, tubulin, and β-actin), metabolic enzymes (GAPDH and neuronal-specific enolase), ion-channel-related proteins (glutamate receptor NR2A), and myelin proteins (2′,3′-cyclic nucleotide 3′-phosphodiesterase) (14). Such findings argue in favor of a neuroprotective role of iNOS-derived NO in EAE, possibly through S-nitrosylation of specific targets in Th17 cells. Nevertheless, since most of the S-nitrosylated proteins identified in the aforementioned study are abundant cellular proteins, in-depth screening with more sensitive proteomic approaches would be required in EAE-infiltrated T cells to search for key S-nitrosylation targets that may influence Th17 differentiation and function. Conversely, the participation of NO in the etiopathology of EAE is not an exclusive hallmark of iNOS. In EAE studies with eNOS-deficient mice, eNOS-derived NO appears to favor the recruitment of T lymphocytes to the CNS, whereas it attenuates EAE exacerbation. Thus, eNOS fulfills proinflammatory and neuroprotective roles during the course of the disease (191). Whether this latter effect involves protein S-nitrosylation remains to be investigated.

Th2 and Tregs. The preferential role for oxidative stress and superoxide in the differentiation toward Th2 phenotypes has recently been highlighted (23, 84). There is also evidence indicating that nitrosothiols may be important for Th2 differentiation and function. Treatment of human bronchial epithelial cells with NO reversibly suppresses proliferation of activated Th2 cells in atopic asthma (35). Moreover, S-nitrosylated proteins are substantially reduced in asthmatic patients, correlating fairly well with the increased GSNOR levels in their bronchoalveolar fluids (140). These findings are supported by studies carried out in an experimental mouse model of asthma, in which mice deficient in the expression of GSNOR were protected against hypersensitivity to the specific allergen and against hyper-reactivity to bronchoconstrictors by increasing the levels of iNOS-derived S-nitrosylated proteins in the lungs, an effect reverted by treatment with NOS inhibitors (141). Furthermore, proteomic studies of T lymphocytes have shown that Trx and other redox control proteins are increased in T cells from asthmatic patients with respect to healthy controls (77).

Several types of Tregs have been implicated in the generation of tolerance to exacerbated inflammatory responses. These include natural and adaptive $CD4^+$ $CD25^+$, $Foxp3^+$ Tregs. Natural Tregs develop in the thymus, whereas adaptive Tregs can be generated by repetitive antigen presentation to naive $CD4^+CD25^-$ T cells by immature or tolerogenic DCs (22, 78, 179). Although recent studies have clearly shown that NO can negatively regulate classical Treg differentiation by inhibiting Foxp3 expression, supposedly through a cGMP-dependent mechanism (16, 93), several lines of evidence do not exclude a role for iNOS-derived NO and S-nitrosylation in the function of Tregs. First, in response to autoantigens, Tregs secrete IFN-γ and induce iNOS-derived NO production by APCs. The APC-derived NO suppresses the proliferation of pathogenic T cells, thereby inhibiting the course of autoimmune diseases (8, 21, 171). Second, DCs expressing a kinase-defective dominant-negative form of Ikappa B kinase-2 (dnIKK2) show an impaired allostimulatory ability to upregulate MHC-II antigens and costimulatory molecules in response to either LPS or CD40 engagement (2). Naive murine T cells stimulated with antigen-loaded immature dnIKK2 DCs differentiate into $CD4^+CD25^-$ Tregs (dnIKK2-Tregs). These Tregs express iNOS, and have the ability to inhibit naive and preactivated T-cell responses in vitro (1). Finally, recent studies demonstrated the existence of a population of Tregs (NO-Treg) generated by NO in a cGMP-independent manner *via* p53-mediated expression of antiapoptotic genes and local secretion of IL-2 (129). NO-Tregs are a subset of T lymphocytes induced by NO together with TcR-mediated activation. They are $CD4^+CD25^+$, $Foxp3^-$, $GITR^+$, and $CD27^+$, and they have a Th2 phenotype that suppresses $CD4^+CD25^-$ effector T cells, exerting their activities in an IL-10-dependent manner. Nonetheless, there is still no consistent evidence that NO-involved Treg functions may take place through S-nitrosylation, beyond a recent report indicating that Tregs express high levels of Trx-1, which may confer resistance to the characteristic oxidative stress of inflammatory foci (120).

Collectively, these diverse lines of evidence suggest that NO can regulate T-cell differentiation and function through a variety of mechanisms, some of them involving the participation of S-nitrosylation, which provides benefits in the course of chronic inflammatory diseases, presumably by disturbing the differentiation and/or function of Th1, Th2, and/or Th17 cells, while the effects on Tregs remain unknown.

S-Nitrosylation as a Therapeutic Agent in Chronic Inflammatory Diseases

Crohn's disease is a chronic inflammatory disease of the gastrointestinal tract, which is probably caused by an imbalance between proinflammatory and anti-inflammatory mediators, and is associated with a permeability disorder of the mucosal barrier (26, 138) (Table 1). A murine model of the disease has been obtained by expressing the herpes simplex virus thymidine kinase (*HSV-Tk*) gene under the control of the astroglial-specific promoter for glial fibrillary acid protein (GFAP). Ganciclovir administration provokes enteric glial ablation in this model, leading to intestinal inflammation and an apparent disruption of the intestinal epithelial monolayer (18). In these mice, GSNO was implicated in promoting the function of the mucosal barrier (154). At low concentrations, and unlike GSH or GSSG, GSNO reduced the intestinal permeability caused by enteric glial cell ablation, and it protected these transgenic mice from intestinal inflammation. ZO-1 is a protein implicated in the formation of tight junctions, and its interaction with actin might directly regulate barrier integrity and permeability. ZO-1 expression increased after exposure to GSNO exposure, a possible means by which GSNO might help maintain mucosal barrier function. This protein may also be regulated by GSNO through PTMs, since ZO-1 protein has a cysteine in a transnitrosylation consensus motif situated in

the C-terminal actin-binding region (154). Future studies might elucidate the mechanisms involved.

Mannose-binding lectin (MBL) is a complement-activating protein involved in innate immune defense. MBL deficit or dysfunction is associated with autoimmune diseases such as lupus erythematosus and rheumatoid arthritis (55, 82). S-nitrosylated MBL was detected in synovial fluid of rheumatoid arthritis patients (59, 65), and there is evidence of the potential harmful effects that S-nitrosylation of MBL might provoke in such patients, including impaired function (mannan binding, complement deposition, bacterial opsonization, agglutination and phagocytosis, and induction of apoptosis) and increased anti-MBL autoantibody generation (59). Given the possible conformational changes in the MBL structure after S-nitrosylation of one or some of its cysteines, it would be of interest to further investigate these potential changes and which cysteine residues might be modified.

Inhaled NO has been used therapeutically in the treatment of acute lung injury, a poorly understood inflammatory syndrome with a significant influence on public health (33). Nevertheless, NO might be not so efficient in treating this disease, since its use may increase the formation of deleterious RNS. By studying lung inflammation in a mouse model of LPS-induced airway injury, it was recently shown that inhalation of ethyl nitrite (ENO), a gas that fosters S-nitrosylation, may decrease lung inflammation and injury in mice by inhibiting epithelial activation in the airways (103). S-nitrosylation homeostasis in the lung was disrupted on LPS treatment, which reduced S-nitrosylation levels and increased NF-κB activation, which was prevented by pretreatment with the S-nitrosylating agent ENO (103).

In conjunction, the findings that managing the levels of S-nitrosylation may be useful in treating those chronic inflammatory diseases in which S-nitrosylation homeostasis is altered considerably are promising. In this regard, S-nitrosylating/denitrosylating agents may represent therapeutic tools for the prevention and/or treatment of this group of diseases. Thus, it is reasonable to expect that in the coming years, these tools could be further developed into more efficient treatments.

Conclusions

S-nitrosylation is increasingly being considered as a signaling mechanism in many cells and systems. In the immune system, several signaling pathways are modulated by this oxidative PTM in the regulation of T-helper cell responses during adaptive immunity, as well as in the innate immunity mediated by phagocytes. Indeed, self-protection mechanisms of macrophages, specialized cells that use NO and RNS production as antimicrobial weapons, have begun to be uncovered. Further work will be required to study these issues in more detail within broader contexts, both from the molecular and pathophysiological points of view, which will benefit from methodological improvements to analyze and quantify the S-nitrosoproteome. There is accumulating evidence of the positive role for nitrosothiols and S-nitrosylation in T-cell regulation, demonstrating beneficial effects in autoimmune disease models, which certainly could be further explored and potentially translated to improve disease treatment.

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Address correspondence to:
Dr. Antonio Martínez-Ruiz
Servicio de Inmunología
Hospital Universitario de La Princesa
C/Diego de León 62
E-28006 Madrid
Spain

E-mail: amartinezr.hlpr@salud.madrid.org

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Abbreviations Used

2-DE = two-dimensional electrophoresis
AICD = activation-induced cell death
APCs = antigen-presenting cells
BST = biotin-switch technique
CNS = central nervous system
COX-2 = cyclooxygenase-2
cPLA2α = cytosolic phospholipase A2α
CRD = carbohydrate-recognition domain
DAG = diacylglycerol
DCs = dendritic cells
dnIKK2 = dominant-negative form of Ikappa B kinase-2
EAE = experimental autoimmune encephalomyelitis
EAU = experimental autoimmune uveitis
ENO = ethyl nitrite
eNOS = endothelial NOS
GFAP = glial fibrillary acid protein
GSH = glutathione
GSNO = S-nitrosoglutathione
GSNOR = S-nitrosoglutathione reductase
HDAC2 = histone deacetylase 2
HSV-Tk = herpes simplex virus thymidine kinase
IFN-γ = interferon-γ
IKK = IκB-kinase
IL-1R = interleukin-1 receptor
iNOS = inducible nitric oxide synthase
IP3 = inositol 1,4,5-triphosphate
IRAK = IL-1R-associated kinase
IRBP = interphotoreceptor retinoid-binding protein
JNK = C-Jun N-terminal kinase
LPS = lipopolysaccharide
MAPK = mitogen-activated protein kinase
MBL = mannose-binding lectin
MS = multiple sclerosis
NO = nitric oxide
PBTs = peripheral blood T cells
PTM = post-translational modification
RNS = reactive nitrogen species
SIRP-α = signal inhibitory regulatory protein α
SNAP = S-nitroso-N-acetylpenicillamine
SP-D = surfactant protein D
TCR = T-cell receptor
TIRAP = Toll/interleukin-1 receptor adaptor protein
TLR = toll-like receptor
TNF = tumor necrosis factor
TNFR = tumor necrosis factor receptor
Tregs = regulatory T cells
Trx = thioredoxin