Central role of nitric oxide in the pathogenesis of rheumatoid arthritis and systemic lupus erythematosus

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Abstract
Nitric oxide (NO) has been shown to regulate T cell functions under physiological conditions, but overproduction of NO may contribute to T lymphocyte dysfunction. NO-dependent tissue injury has been implicated in a variety of rheumatic diseases, including systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). Several studies reported increased endogenous NO synthesis in both SLE and RA, and recent evidence suggests that NO contributes to T cell dysfunction in both autoimmune diseases. The depletion of intracellular glutathione may be a key factor predisposing patients with SLE to mitochondrial dysfunction, characterized by mitochondrial hyperpolarization, ATP depletion and predisposition to death by necrosis. Thus, changes in glutathione metabolism may influence the effect of increased NO production in the pathogenesis of autoimmunity.

Basic functions of nitric oxide
Nitric oxide (NO) is a short-lived signaling molecule that plays an important role in a variety of physiologic functions, including the regulation of blood vessel tone, inflammation, mitochondrial functions and apoptosis [1,2]. NO was originally identified as endothelium-derived relaxant factor based on the observations of Furchgott and Zawadzki [3]. They observed that acetylcholine-induced blood vessel relaxation occurred only if the endothelium was intact. Some years later, the endothelium-derived relaxant factor was identified as NO [4]. NO is synthesized from L-arginine by NO synthetases (NOSs): neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS) [5]. NO also serves as a potent immunoregulatory factor, and influences the cytoplasmic redox balance through the generation of peroxynitrite (ONOO-) following its reaction with superoxide (O2-) [6]. In addition, NO regulates signal transduction by regulating Ca2+ signaling, by regulating the structure of the immunological synapse, or through the modification of intracellular proteins, such as by interactions with heme groups (Figure 1). Here we summarize the effects of NO on T lymphocyte functions in both systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA).

NO regulates mitochondrial membrane potential in human T cells [7], and may both stimulate and inhibit apoptosis [8]. It was shown to inhibit cytochrome c oxidase, leading to cell death through ATP depletion (Figure 1). In addition, NO was shown to regulate mitochondrial biogenesis in U937 and HeLa cells and adipocytes through the cGMP-dependent peroxisome proliferator-activating receptor α coactivator 1α [9]. According to our earlier work, NO regulates mitochondrial biogenesis in human lymphocytes as well [10]. Nitrosylation of sulfhydryl groups represents an important cGMP-independent, NO-dependent post-translational modification. Several important signal transduction proteins are potential targets of S-nitrosylation, such as caspases and c-Jun-N-terminal kinase (JNK) [11,12].

The role of nitric oxide in T cell activation and differentiation
NO regulates T lymphocyte function in several ways: T cell activation is associated with NO production and mitochondrial hyperpolarization (MHP) [13]. According to our previous data, eNOS and nNOS are expressed in human peripheral blood lymphocytes and both are up-regulated several times following T cell activation [13]. TCR stimulation induces Ca2+ influx and, through inositol-1,4,5-trisphosphate (IP3), the release of Ca2+ from intracellular stores. The IP3 inhibitor 2-APB (2-aminophosphorylidene borane) decreases T-cell-activation-induced Ca2+ and NO production, and NO

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treatment of T lymphocytes leads to an increase in mitochondrial and cytoplasmic Ca\(^{2+}\) levels. In contrast, the NO chelator C-PTIO (carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide) powerfully inhibits the T-cell-activation-induced Ca\(^{2+}\) response, NO production and MHP, indicating that T cell receptor (TCR)-activation-induced MHP is mediated by NO [13].

A central event in the antigen-specific interaction of T cells with antigen-presenting cells is the formation of the immunological synapse, in which the TCR complex and the adhesion receptor LFA-1 (leukocyte function-associated antigen 1) are organized in central and peripheral supramolecular activation clusters. eNOS was shown to translocate with the Golgi apparatus to the immune synapse of T helper cells engaged with antigen-presenting cells [14] (Figure 1). Overexpression of eNOS was associated with increased phosphorylation of the CD3\(\zeta\) chain, ZAP-70, and extracellular signal-regulated kinases, and increased IFN-\(\gamma\) synthesis, but reduced production of IL-2. These data indicate that eNOS-derived NO selectively potentiates T cell receptor signaling to antigen at the immunological synapse [14].

Following activation, CD4 T cells proliferate and differentiate into two main subsets of primary effector...
cells, T helper 1 (Th) and Th2 cells, characterized by their specific cytokine expression patterns [15]. The Th1/Th2 balance is considered to be essential in chronic inflammatory diseases. NO selectively enhances Th1 cell proliferation [16] and represents an additional signal for the induction of T cell subset response. According to our data, the NO precursor NOC-18 elicited IFN-γ production, whereas the NO synthase inhibitors NG-monomethyl-L-arginine and nitronidazole both inhibited its production, suggesting a role for NO in regulating IFN-γ synthesis [17]. NO preferentially promotes IFN-γ synthesis and type Th1 cell differentiation by selective induction of IL-12Rβ2 via cGMP. Together, these data indicate that NO has a crucial role in the regulation of Th1/Th2 polarization.

Nitric oxide regulates T lymphocyte activation in systemic lupus erythematosus

Considerable evidence supports that NO production is increased in SLE; for example, serum nitrite and nitrate levels were recently reported to correlate with disease activity and damage in SLE [18]. According to our previous work, NO plays a crucial role in T cell dysregulation in SLE [19-21]. Activation-induced rapid Ca2+ signals are higher in T cells from patients with SLE [22]; in contrast, the sustained Ca2+ signal is decreased in these lupus T cells. Interestingly, the mitochondrial membrane potential is permanently high in lupus T cells [23-25]. Lupus and normal T cells produce comparable amounts of NO, but monocytes from lupus patients generate significantly more NO than normal monocytes. As it is a diffusible gas, NO produced by neighboring cells may affect T cell functions. Accordingly, NO produced by monocytes contributes to lymphocyte mitochondrial dysfunction in SLE [10]. Peripheral blood lymphocytes from SLE patients contain enlarged mitochondria, and as there are microdomains between mitochondria and the endoplasmic reticulum and because mitochondria may also serve as Ca2+ stores, this increased mitochondrial mass may alter Ca2+ signaling in SLE [10,26]. Although NO production was found to be increased in both lupus [10] and RA [27], MHP was confined to lupus T cells [10,13,28,29]. This difference may be attributed to the depletion of intracellular glutathione (GSH) in SLE but not in RA or healthy controls [28]. Indeed, low GSH predisposes to MHP in human T cells, as originally described by Banki and colleagues [30]. Increased exposure to IFN may contribute to the increased NO production of lupus monocytes [31].

NO regulates mammalian target of rapamycin activity and TCRζ expression in SLE

The mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase and a sensor of the mitochondrial transmembrane potential that regulates protein synthesis, cell growth, cell proliferation and survival [32]. The activity of mTOR is increased in lupus T cells [29] (Table 1); furthermore, NO regulates mTOR activity, which leads to enhanced expression of HRES-1/Rab4, a small GTPase that regulates recycling of surface receptors through early endosomes [29,33]. HRES-1/Rab4 overexpression inversely correlates with TCRζ protein levels. TCR/CD3 expression is regulated by TCRζ, and diminished ζ chain expression disrupts TCR transport and function [34]. The TCR ζ chain is deficient in lupus T cells [35,36], although this deficiency has been shown to be independent of SLE disease activity [37,38]. Sequencing of genomic DNA and TCRζ transcripts showed mutations in the coding region of TCRζ from lupus T cells [39]. There is a direct interaction between HRES-1/Rab4, CD4 and TCRζ. Rapamycin treatment of lupus patients reversed the TCRζ deficiency of lupus T cells, and normalized T-cell-activation-induced calcium fluxing [29]. These data suggest that NO-dependent mTOR activation induces the loss of TCRζ in lupus T cells through HRES-1/Rab4. Several earlier findings indicate that decreased TCRζ chain expression may also be independent of NO in SLE [40-44].

Consequences of increased nitric oxide production in rheumatoid arthritis

Several studies in patients with RA have documented evidence for increased endogenous NO synthesis, suggesting that overproduction of NO may be important in the pathogenesis of RA. The inflamed joint in RA is the predominant source of NO [45,46]. Several investigators found correlations between serum nitrite concentration and RA disease activity or radiological progression while others did not find such correlations [47,48]. NOS polymorphism has been observed in RA [49]. iNOS is regulated at the transcriptional level, while eNOS and nNOS are regulated by intracellular Ca2+. Several different cell types are capable of generating NO in the inflamed synovium, including osteoblasts, osteoclasts, macrophages, fibroblasts, neutrophils and endothelial cells [50-52]. NOS inhibition was reported to decrease disease activity in experimental RA [53]. We have shown recently that T cells from RA patients produce more than 2.5 times more NO than healthy donor T cells (P < 0.001) [27]. Although NO is an important physiological mediator of mitochondrial biogenesis, mitochondrial mass is similar in both RA and control T cells (Table 1). By contrast, increased NO production is associated with increased cytoplasmic Ca2+ concentrations in RA T cells (P < 0.001). Furthermore, in vitro treatment of human peripheral blood lymphocytes or Jurkat cells with TNF increases NO production (P = 0.006 and P = 0.001, respectively), whilst infliximab treatment
of RA patients decreases T-cell-derived NO production within 6 weeks of the first infusion ($P = 0.005$) [27]. Increased NO production of monocytes is associated with increased mitochondrial biogenesis in lupus T cells, while increased NO production of T cells is not associated with increased mitochondrial mass in RA. Monocytes express iNOS, while lymphocytes express both eNOS and nNOS. Although NO is generated more rapidly via the eNOS or nNOS than the iNOS pathway, iNOS can generate much larger quantities of NO than eNOS and nNOS. Thus, the lower amount of NO generated by T cells compared to monocytes may explain the differences in T lymphocyte mitochondrial biogenesis that we observed for lupus and RA T cells.

iNOS knockout mice are resistant to IL-1-induced bone resorption, suggesting that NO plays a central role in the pathogenesis of bone erosions in RA [51,54]. TNF blockade decreases iNOS expression in human peripheral blood mononuclear cells [55]. *Tripterygium wilfordii* Hook F (TWHF) was also reported to be effective in the treatment of experimental arthritis [56]. The specific inhibition of iNOS by TWHF is probably responsible for the anti-inflammatory effects of this medicinal plant. NO treatment may lead to necrosis rather than apoptosis by decreasing intracellular ATP levels. The release of intracellular antigens through necrosis may accelerate autoimmune reactions leading to chronic inflammation [57,58].

### Oxidative stress and TCRζ expression in RA T cells - the possible role of NO

Reduced GSH levels may contribute to the hyporesponsiveness of T cells from synovial fluid of RA patients [59,60]. The expression of the TCR ζ chain protein is decreased in synovial fluid T cells of RA patients, similar to lupus T cells, which may contribute to the above-mentioned hyporesponsiveness of the synovial fluid T cells [61]. TNF-α treatment decreases TCR ζ chain expression of T cells [62] in a GSH-precursor-sensitive way, showing the role of redox balance in the regulation of TCR ζ chain expression. TCRζ overexpression does not restore signaling in TNF-α-treated T cells [63]. Increased NO production may alter redox balance through generating peroxynitrite following its reaction with superoxide. In this way NO may contribute to the decreased TCR ζ chain expression of T lymphocytes from synovial fluid [61]. Importantly, FcγR gamma substitutes for the TCR ζ chain in SLE T cells [64], which may explain the enhanced T-cell-activation-induced Ca²⁺ fluxing. The potential role of NO in the regulation of FcγR gamma expression clearly needs further investigation.

### Th17 and regulatory T cells

Recently, the Th1/Th2 paradigm has been updated following the discovery of a third subset of Th cells, known as Th17 cells. Th17 cells have been identified as cells induced by IL-6 and TGF-β and expanded by IL-23 [65]. Similarly to Th1 and Th2 subsets, Th17 development relies on the action of a lineage-specific transcription factor. Th17 cells have emerged as an independent subset because their differentiation was independent of the Th1 and Th2 promoting transcription factors T-bet, STAT1, STAT4 and STAT6. ROR-γt, RORα and STAT3 appear to be critical for the development of Th17 cells. Th17 cells produce IL-17 and are thought to clear extracellular pathogens that are not effectively handled by either Th1 or Th2 cells, and have also been strongly implicated in allergic diseases [66]. In addition to IL-17, Th17 cells produce other proinflammatory cytokines such as IL-21 and IL-22. Increased levels of IL-17 have been observed in patients with RA. Indeed, IL-17 can directly and indirectly promote cartilage and bone destruction. IL-17-deficient mice develop attenuated collagen-induced arthritis. The role of NO in IL-6- and TGF-β-induced Th17 cell differentiation has not been studied yet.

Regulatory T cells (Tregs) represent a subset of T cells involved in peripheral immune tolerance. There are at least three major types of Tregs with overlapping functions: Th3, Treg1, and CD4⁺CD25⁺ Tregs. CD4⁺CD25⁺ Tregs (naturally occurring cells or nTREGs) are the best characterized, principally because it is relatively easy to obtain large numbers of these cells. Tregs seem to have

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**Table 1. Nitric oxide-induced T cell functions in systemic lupus erythematosus and rheumatoid arthritis**

| Altered T cell function                  | SLE                                      | RA                                      |
|------------------------------------------|------------------------------------------|------------------------------------------|
| Mitochondrial hyperpolarization and biogenesis | Higher [10]                              | Normal [27]                             |
| T lymphocyte NO production               | Normal [10]                              | Increased [27]                          |
| TCR-induced rapid and sustained Ca²⁺ signal | Rapid-increased, sustained-decreased [10] | Normal [22]                             |
| TCR expression                           | Decreased [34]                           | Decreased [61]                          |
| mTOR activity                            | Increased [29]                           | Not known                               |
| ATP level                                | Decreased [28]                           | Normal [28]                             |
| Monocyte NO production                   | Increased [10]                           | Increased [46]                          |

mTOR, mammalian target of rapamycin; NO, nitric oxide; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; TCR, T cell antigen receptor.
an impaired regulatory function in RA. It was recently reported that NO, together with anti-CD3, induces the proliferation and sustained survival of mouse CD4+CD25− T cells, which become CD4+CD25+ but remained Foxp3−. This previously unrecognized population of Tregs (NO-Tregs) downregulated the proliferation and function of freshly purified CD4+CD25− effector cells in vitro and suppressed colitis- and collagen-induced arthritis in mice in an IL-10-dependent manner [67]. The existence of human NO-Tregs has not been investigated yet. Although NO profoundly alters T cell activation and Th1/Th2 balance, the precise role of NO in Th17 and Treg differentiation is not known.

Conclusion

Whilst NO plays a central role in many physiological processes, its increased production is pathological. NO mediates many different cell functions at the site of synovial inflammation, including cytokine production, signal transduction, mitochondrial functions and apoptosis (Table 1). The effects of NO depend on its concentration. Increased NO production plays an important role in the pathogenesis of both SLE and RA. Further studies are needed to determine the cellular and molecular mechanisms by which NO regulates immune cell functions. NO inhibition may represent a novel therapeutic approach in the treatment of chronic autoimmune diseases.

Abbreviations

eNOS = endothelial NO synthase; GSH = glutathione; IFN = interferon; IL = interleukin; iNOS = inducible NO synthase; IP3 = inositol-1,4,5-triphosphate; MHP = mitochondrial hyperpolarization; mTOR = mammalian target of rapamycin; nNOS = neuronal NO synthase; RA = rheumatoid arthritis; SLE = systemic lupus erythematosus; TCR = T cell antigen receptor; TGF = transforming growth factor; Th = T helper; TNF = tumor necrosis factor; Treg = regulatory T cell; TWHF = Tripterygium wilfordii Hook F.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

This work has been supported by grants RO1 AI 048079 and AI 072678 from the National Institutes of Health, the Alliance for Lupus Research, the Central New York Community Foundation, as well as OTKA K77537 and OTKA K73247. György Nagy is a Bolyai Research fellow.

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Published: 28 June 2010

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