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The role of nitric oxide in abnormal T cell signal transduction in systemic lupus erythematosus

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Abstract

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by production of antinuclear autoantibodies and diverse array of clinical manifestations. T cells from patients with SLE have been shown to be activated in vivo and provide help to autoreactive B cells. Lupus T cells exhibit enhanced spontaneous and diminished activation-induced apoptosis and predisposition to necrosis. Persistent mitochondrial hyperpolarization and ATP depletion – associated with significantly increased mitochondrial mass – characterize T lymphocyte dysfunction in SLE. In addition to cell death abnormalities, mitochondrial dysfunction is associated with altered signal transduction through the T cell receptor and Ca^{2+} fluxing. Exposure of normal T cell to nitric oxide induces mitochondrial hyperpolarization and biogenesis and regenerates the Ca^{2+} signaling profile of lupus T cells. This article reviews a novel understanding of the role of nitric oxide in signal transduction and cell death abnormalities in SLE.

Introduction

Systemic lupus erythematosus (SLE) is a polysystemic autoimmune disease, with unknown origin, characterized by waxing and waning course and the involvement of multiple organs including skin, kidneys, and central nervous system. Current evidence suggests that a central role in initiating disease is played by underlying defects in T lymphocyte antigen receptor-mediated signaling that lead to B cell hyper-responsiveness, increased spontaneous and decreased activation-induced apoptosis, skewed cytokine production, and breakdown of immunological tolerance [1-6].

The mitochondrion lies at the heart of cell life and cell death, the integrity of mitochondrial function is fundamental to cell survival. It follows that disturbances of mitochondrial function will lead to disruption of cell function, expressed as disease. It is widely accepted that apoptotic abnormalities contribute to the pathogenesis of SLE [1-3]. Recently, enhanced spontaneous and diminished activation-induced apoptosis has been found to be associated with abnormal TCR activation, increased mitochondrial biogenesis, persistent mitochondrial hyperpolarization (MHP), ATP depletion, and overproduction of nitric oxide (NO). This

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Abbreviations: SLE, systemic lupus erythematosus; ITAM, IgR family tyrosine based activation motif; LCK, lymphocyte-specific protein tyrosine kinase; LAT, linker for activation; IP₃, inositol-1,4,5-triphosphate; PI₃K, phosphatidylinositol 3-hydroxyl kinase; IFN, interferon; HMGP, high mobility group protein; ROI, reactive oxygen intermediate; $\Delta\psi_m$, mitochondrial transmembrane potential; MHP, mitochondrial hyperpolarization; NOS, NO synthase; nNOS, neuronal NOS; eNOS, endothelial; iNOS, iNOS inducible NOS; MPT, mitochondrial Ca^{2+} -induced permeability transition; MPA, mycophenolic acid; TCR, T cell antigen receptor; ZAP-70, ζ -associated protein-70.

review will focus on the central role of NO and Ca^{2+} in the altered T cell activation and mitochondrial dysfunction in SLE.

Abnormal TCR activation and Ca^{2+} signaling in SLE

Engagement of the T cell antigen receptor (TCR) leads to activation of multiple protein tyrosine and serine kinases resulting in phosphorylation of intracellular substrates. During T cell activation phospholipase, C- γ 1 catalyzes hydrolysis of phosphatidylinositol 4,5 biphosphate to inositol 1,4,5 trisphosphate (IP_3) and diacylglycerol. IP_3 binds to its receptor in the endoplasmic reticulum and releases Ca^{2+} to the cytosol by opening Ca^{2+} channels, followed by capacitative Ca^{2+} entry through the plasma membrane [7]. Ca^{2+} serves as an intracellular messenger regulating different signaling pathways that lead to T cell activation and proliferation such as IL-2 synthesis, PKC activation, activation of calmodulin and calmodulin-dependent protein kinases, calcineurin, and NFAT [8,9]. Several defects of the signaling cascade in SLE T cells have been discovered.

In contrast to normal and rheumatic disease control T cells, TCR ζ chain protein is deficient in T cells of most SLE patients [10,11]. T cell ζ chain deficiency is persistent over time and is independent of SLE disease activity and treatment [12,13]. Cloning and sequencing of genomic DNA and TCR ζ transcripts revealed widely divergent mutations and silent polymorphism in the coding region in lupus T cells. Alternatively spliced forms of the 8 exons of TCR ζ chain transcripts were observed at increased frequency. Nontranslated downstream region of the ζ chain gene is shorter, which may account for decreased messenger RNA stability. Several of the mutations were located in the 3 immunoreceptor tyrosine activation motifs, or the GTP binding domain, and this could lead to functional alterations in the TCR zeta chain [2,14].

The regulation of lymphocyte-specific protein tyrosine kinase (LCK) is critical for maintaining the resting state of T cells and for initiating the activation of signaling cascades [15]. LCK kinase activity is regulated by the phosphorylation of two important tyrosine sites: an autophosphorylation site (Tyr394) and an opposing negative regulatory site (Tyr505). Phosphorylation of the negative regulatory site induces the inactive or “closed” LCK conformation. There is good evidence that LCK tyrosine phosphorylation is controlled by the reciprocal action of the protein tyrosine phosphatase (PTP) CD45 and the PTK C-terminal Src kinase (CSK). CD45 can positively and negatively regulate the activity of LCK by controlling the phosphorylation of the regulatory tyrosine residues (Tyr394 and Tyr505) [15]. In the resting T cell, CD45 maintains LCK in a primed state ready to respond to TCR activation by dephosphorylating Tyr505. *P56^{lck}* mRNA and protein levels are reduced in T cells from SLE patients [16-18]. T lymphocytes from patients with SLE have reduced expression of LCK that is independent of treatment regime but is associated with disease activity [19]. The expression of *p56^{lck}* was inversely correlated with apoptosis of fresh and cultured SLE PBLs, especially in anti-DNA negative patients [17]. Although *P56^{lck}* expression was reduced, the phosphorylation of *P56^{lck}* was increased in the patients with active SLE [18]. CD45 PTP-ase activity was reduced in patients with active SLE [20]. Protein tyrosine phosphatase (PTP-ase) activity of cell lysates immunoprecipitated by CD45 monoclonal antibody is decreased in SLE patients. The expression of CD45RA and CD45RO on PBL was comparable between normal and SLE T cells. CD45 was differentially expressed and its localization into lipid rafts was increased in T cells from patients with SLE [21,22].

Activation of T cells through the TCR initiates rapid increase in cytoplasmic and mitochondrial Ca^{2+} levels (within 5–10 min), followed by a plateau phase, lasting at least 48 h [23]. Lupus T cells exhibit an enhanced rapid phase of cytoplasmic Ca^{2+} signaling in

response to TCR stimulation [24,25]. This initial surge in Ca^{2+} is due to the release of Ca^{2+} from internal stores such as mitochondria [26]. The altered Ca^{2+} signal does not correlate with disease activity, or organ system involvement, indicating that the detected increased Ca^{2+} response may be important for pathogenesis of the disease.

Inhibition of Ca^{2+} ATP-ase results in emptying of the intracellular Ca^{2+} stores, and to capacitative Ca^{2+} influx from extracellular space. The sarcoplasmic and endoplasmic Ca^{2+} ATP-ase inhibitor thapsigargin-induced Ca^{2+} signal is similar in both SLE and normal T cells [24]. Moreover, Ca^{2+} ionophore ionomycin-induced Ca^{2+} signal was similar in both SLE and normal T cells, indicating that increased Ca^{2+} response in lupus T cells may not derive from altered influx across the cell membrane. The amount of Ca^{2+} that was released from the intracellular stores was higher in lupus than control T cells, indicating the possible role of increased intracellular Ca^{2+} stores in lupus [24]. The production of inositol phosphate metabolites in SLE cells is comparable with normal cells. Similar to control, SLE CD4^{+} cells display higher Ca^{2+} response than CD8^{+} positive cells. In SLE, both CD4^{+} and CD8^{+} positive cells displayed higher Ca^{2+} response [24].

Interestingly, baseline cytoplasmic Ca^{2+} levels are increased in SLE T cells [26]. T cell activation-induced rapid Ca^{2+} signal is followed by a plateau phase, lasting at least 48 h. While the early phase of Ca^{2+} signal was enhanced, sustained elevation of CD3/CD28 -induced Ca^{2+} signaling was markedly reduced in lupus T cells [26]. Sustained Ca^{2+} signal mediates activation of PKC, calcineurin, NFAT, and production of IL-2. Thus, deficiency of sustained Ca^{2+} signaling may contribute to reduced IL-2 production of lupus T cells.

Persistent mitochondrial hyperpolarization and ATP depletion in SLE

The inner mitochondrial membrane is largely impermeant and forms the major barrier between the cytosol and the mitochondrial matrix [27]. Mitochondrial respiration establishes a large potential gradient across the inner mitochondrial membrane. At complexes I, III, and IV, the free energy released by the fall in redox potential of the passing electrons is used to translocate protons from the mitochondrial matrix into the intermembrane space [27]. This process generates an electrochemical potential gradient across the inner mitochondrial membrane, which is estimated to be 180 mV, negative inside the matrix. During oxidative phosphorylation, the energy is generated by the electron transport chain and stored as an electric gradient. The mitochondrial membrane potential lies at the heart of all the major bioenergetic functions of the mitochondrion from the manufacture of ATP to accumulation of Ca^{2+} . The energy stored in the transmembrane potential is used by F_1F_0 -ATP-ase to generate ATP from ADP. Virtually all energy-consuming processes in the cells utilize high-energy phosphates such as ATP. Unlike glycolysis, which takes place in the cytoplasm, oxidative phosphorylation, that takes place in the mitochondria, is the most effective way of ATP synthesis in mammalian cells [27].

Mitochondria have been recently recognized as a key controller of programmed cell death besides being the textbook “powerhouse of the cell”. Disruption of the mitochondrial transmembrane potential ($\Delta\Psi_m$) has been proposed as the point of no return in apoptotic signaling [28, 29]. Fas-induced apoptosis is preceded by mitochondrial hyperpolarization (MHP) and increased production of reactive oxygen intermediates (ROI). Mitochondrial hyperpolarization was observed as early as 20 min after stimulation with anti-Fas antibody [30]. Mitochondrial transmembrane potential ($\Delta\Psi_m$) may be elevated for several hours during T cell activation or cell death. Interleukin-3 (IL-3), interleukin-10 (IL-10), transforming growth factor- β_1 (TGF- β_1), and interferon-gamma (IFN- γ) were also found to elicit transient $\Delta\Psi$ elevation [31-34].

Lupus T cells were recently discovered to exhibit persistent MHP or elevation of the mitochondrial transmembrane potential ($\Delta\Psi_m$), increased ROI levels and ATP depletion [35]. Given the fundamental role of mitochondria as ATP generators and the requirement of cells for ATP, it seems self-evident that severe damage of mitochondria will cause cell death. T lymphocytes of patients with SLE showed increased spontaneous and decreased activation-induced apoptosis [35,36]. Upon T cell activation, cell death preferentially occurred via necrosis rather than apoptosis in patients with SLE. ATP levels and ATP/ADP ratios were profoundly diminished in lupus PBL which account for predisposition to necrosis in lupus T cells [35,37].

Calcium and mitochondria

While the principle function of the mitochondrial potential is to drive ATP synthesis, it also provides the major mechanism to handle Ca^{2+} . Exposure of isolated mitochondria to artificially created Ca^{2+} pulses, like those seen in the cytosol of the cells, led to the discovery of mitochondrial Ca^{2+} uptake. Intramitochondrial Ca^{2+} activates the electron transport chain, adenine nucleotide translocase (ANT), and F_1F_0 -ATP-ase [38].

The three major rate limiting enzymes of the citric acid cycle (isocitrate dehydrogenase, citrate synthase, ketoglutarate dehydrogenase) are all upregulated by Ca^{2+} , thereafter mitochondrial Ca^{2+} uptake increases mitochondrial ATP production [38,39]. Ca^{2+} is taken up through the mitochondrial inner membrane through a uniporter. Now it seems clear that physiological Ca^{2+} signals in most cells are associated with some measure of Ca^{2+} accumulation in the mitochondria. Under pathological conditions, mitochondrial Ca^{2+} accumulation may also play a key role in determining the outcome, acting as a trigger to pathophysiological events that may dictate the death of the cell. Although mitochondrial ATP production can be upregulated by increasing concentrations of ADP and Pi as well [40], oxidative phosphorylation can be stimulated more rapidly by pulses of Ca^{2+} than ADP [38,41].

Mitochondrial Ca^{2+} uptake is primarily driven by the electrochemical potential gradient, established by the mitochondrial membrane potential and a relatively low intramitochondrial Ca^{2+} concentration. The accumulation of Ca^{2+} into mitochondria has major functional consequences for the mitochondria and for the cell. Since re-equilibration of mitochondrial Ca^{2+} is relatively slow, mitochondria effectively integrate cytosolic Ca^{2+} signals over time [42]. Persistent mitochondrial hyperpolarization (MHP), ATP depletion and predisposition to necrosis suggested altered mitochondrial Ca^{2+} handling by lupus T cells.

Indeed, baseline mitochondrial Ca^{2+} levels are significantly increased while CD3/CD28 costimulation-induced sustained mitochondrial Ca^{2+} signals were delayed in lupus T cells [26]. Moreover, mitochondria constitute the primary source of reactive oxygen intermediate (ROI) generation in lymphocytes [2]. This is because a consequence of the action of oxidative phosphorylation is the generation of unpaired electrons. The interaction of these electrons with O_2 results in the generation of superoxide ions, highly reactive free radical species. These are readily interconverted to other radical species, such as hydroxyl ions (OH^-) and H_2O_2 . ROI may cause lipid peroxidation, and damage to cell membranes and to DNA, so that mitochondria represent not only a major source of ROI generation, but also a major target of ROI-induced damage [27]. The leak of unpaired electrons is increased at higher $\Delta\Psi_m$, thus, persistent MHP is a likely cause of increased ROI production, i.e. oxidative stress in lupus T cells.

Role of nitric oxide in T cell activation, mitochondrial biogenesis, and hyperpolarization

Crosslinking of the TCR has been associated with elevation of the cytosolic Ca^{2+} , ROI, and NO production and mitochondrial hyperpolarization [43]. NO chelator C-PTIO (carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide) profoundly inhibited TCR crosslinking-induced mitochondrial hyperpolarization, cytoplasmic and mitochondrial Ca^{2+} response, ROI production, and NO levels, suggesting that TCR activation-induced MHP is mediated by NO. In addition, NO signal was required for CD3/CD28-induced Ca^{2+} fluxing.

NO is a highly diffusible gas that is synthesized from the amino acid L-arginine, by a family of enzymes termed NO synthases (NOS). Three distinct isoforms of NOS are known. Two of these, neuronal NOS (nNOS) and endothelial NOS (eNOS) isoforms, were named after the tissues in which they were first identified. There is also an inducible isoform (iNOS) which generated NO in larger quantities during immunological defense reactions [44]. NO is widely used as a signaling molecule throughout the body, carrying out numerous roles but most notably regulating local vascular tone and blood flow. Western blot analysis revealed expression of eNOS and nNOS and absence of iNOS in human PBL. eNOS and nNOS protein levels were stimulated up to 15-fold by CD3/CD28 costimulation [43,45].

Mitochondrial complex IV (cytochrome oxidase) has a high affinity for NO, which competes with oxygen and inhibits mitochondrial respiration. NO promoted mitochondrial hyperpolarization, ATP depletion, and relative resistance to apoptotic stimuli in astrocytes, lymphocytes, and Jurkat cells [46,47]. Moreover, pretreatment of normal T cells with NO resulted in elevation of mitochondrial and cytoplasmic Ca^{2+} level [43]. Recently, Fas-induced apoptosis was found to be associated with an early, concentration-dependent NO production in Jurkat cells [48]. Low concentrations of NO may specifically inhibit cytochrome *c* oxidase, leading to ATP depletion [49].

NO has newly been recognized as a key signal for mitochondrial biogenesis. This operates through the cGMP-dependent peroxisome proliferator-activating receptor γ coactivator-1 α , a master regulator of mitochondrial biogenesis. NO was shown to induce mitochondrial biogenesis in brown adipocytes, U937 and HeLa cells, and human lymphocytes [26,50].

NO appears to be a bifunctional modulator of cell death capable of either stimulating or inhibiting apoptosis [51-53]. NO reacts rapidly with superoxide (O_2^-) to produce peroxynitrite (ONOO^-), which may act as an oxidant itself, or isomerize to nitrate. NO can induce apoptosis in a variety of cell lines including macrophages, thymocytes, T cells, and myeloid cells. A site for NO modulation of the apoptotic process that has received little attention is the controlled release of cytochrome *c* from the mitochondria [54]. The release of cytochrome *c* from mitochondria leads to activation of caspases 9 and 3. The proapoptotic effect of NO may be connected to its effect on mitochondrial respiration. NO – through its interaction with components of the electron-transport chain – might function not only as a physiological regulator of cell respiration, but also to augment the generation of reactive oxygen species by mitochondria, and thereby trigger mechanisms of cell survival or death [55].

The role of interferon IFN- α in the pathogenesis of SLE

CD14 monocytes from SLE patients can differentiate into dendritic cells (DCs) which can to induce proliferation of allogenic CD4 T cells [56-58]. Most patients with SLE have increased IFN serum levels and majority of active SLE patients were reported to have increased transcription of IFN regulated genes (IFN biosignature) [59]. IFN- α appears to be the main

factor driving unabated DC differentiation/activation in SLE [60]. In SLE, the plasmacytoid dendritic cells release large amounts of IFN, which induces DC maturation. The mature DCs that have captured apoptotic cells activate, rather than silence autoreactive T cells, leading to increased production of autoantibodies by plasma cells (Fig. 1). Calreticulin and high mobility group protein (HMGP1) released by necrotic T cells promote expansion and differentiation of dendritic cells [31]. Although treatment of normal monocytes or mononuclear cells in vitro with IFN caused a dose-dependent increase in monocyte iNOS activity, NO production, and increased expression of iNOS protein [61], NO negatively regulates the expression and activity of IFN in monocytes [62].

Nitric oxide induced mitochondrial biogenesis mediated altered Ca^{2+} signaling profile of lupus T cells

Considerable evidence supports a role of increased NO production in SLE. Oral administration of either N^G -monomethyl-L-arginine, a nonspecific NOS inhibitor, or L- N^6 -(1-iminoethyl) lysine (L-NIL), an iNOS-specific inhibitor, prevented the development of glomerulonephritis, reduced the intensity of arthritis, and lowered renal pathology scores in MRL-*lpr/lpr* mice [63]. However, MRL/*lpr* iNOS knockout mice develop renal disease similar to the wild-type animal [64]. Belmont and colleagues reported elevated serum nitrite levels, an in vivo marker of NO production, in lupus patients [65,66]. Serum nitrite levels correlated with disease activity and anti-DNA antibody levels. Spleen and peritoneal cells from MRL/*lpr* mice produced significantly more NO than those from the control mice when cultured with interferon (IFN) γ and lipopolysaccharide (LPS) in vitro [67,68].

Persistent MHP has been associated with increased mitochondrial mass of T lymphocytes in patients with SLE [26]. Electron microscopy revealed that T cells of lupus patients contain more mitochondria than normal T cells, in addition lupus lymphocytes harbor several-fold enlarged mitochondria, termed megamitochondria [26]. Mitochondria can take up, store, and release Ca^{2+} , thereafter increased mitochondrial mass may account for altered Ca^{2+} handling in SLE (Fig. 1).

In HeLa cell, transfected with mitochondrially targeted aequorin, permeabilized and exposed to added Ca^{2+} buffer solutions, net mitochondrial Ca^{2+} accumulation was only detectable if the added Ca^{2+} reached concentration higher than 3 μM , while Ca^{2+} signal evoked by IP3 mobilizing agents was far more effective at raising Ca^{2+} . This observation led to the suggestion that mitochondria must be positioned at privileged sites, close to the ER Ca^{2+} release sites. Indeed, mitochondria form contact sites with ER through local microdomains allowing Ca^{2+} exchange. This mechanism allows mitochondria to play a significant role in shaping the spatiotemporal patterning of cytoplasmic Ca^{2+} signals [69]. This is in accordance with the observation that Ca^{2+} released from the intracellular stores was higher in lupus than control T cells [24]. Moreover, treatment of normal T cells with NO donor mimicked the altered Ca^{2+} signal observed in SLE [26].

Although SLE T cells and normal T cells produce comparable amount of NO, lupus monocytes were found to produce significantly higher amount of NO than normal monocytes, [70,71]. Activated monocytes play a crucial role in the autoimmune response in SLE. In comparison to control monocytes, lupus monocytes increased $\Delta\Psi_m$ and Ca^{2+} of normal T cells after coculture [26], suggesting that nitric oxide produced by monocytes is responsible for mitochondrial dysfunction in SLE.

Future directions

Understanding the precise role of NO in the pathogenesis of SLE may help answer a number of outstanding questions: Does overproduction of NO have a role in the TCR ζ -chain deficiency in SLE? Is there a cross regulation between NO and IFN in lupus? Does increased mitochondrial number and megamitochondria characterize specific T cell subsets? Could NO chelators or NO synthase inhibitors normalize NO production by lupus monocytes and restore $\Delta\Psi_m$, Ca^{2+} handling, and cell death pathway selection by lupus T cells and provide a therapeutic benefit for patients with SLE?

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Fig. 1.

Schematic diagram of intercellular signals underlying mitochondrial dysfunction of lupus T cells. Increased NO production by monocytes generates mitochondrial biogenesis in T cells, leading enhanced mitochondrial Ca^{2+} content and altering Ca^{2+} signaling profile of lupus T cells. NO induces mitochondrial hyperpolarization and ATP depletion thus contributing to relative resistance to apoptotic stimuli and increased necrosis. Necrotic T cells release factors such as calreticulin and high mobility group protein (HMGP1) that promote expansion and differentiation of dendritic cells. Dendritic cells (DC) produce increased amounts of IFN that, in turn, enhances monocyte iNOS activity and NO production. Positive feed back between necrosis-prone T cells, NO-producing monocytes, and enhanced IFN production by dendritic cells create a vicious cycle and lead to persistent inflammation in SLE.