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# Neutrophils recruitment during sepsis: Critical points and crossroads

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## 1. ABSTRACT

The mechanisms that initiate an inflammatory systemic response to a bacterial infection lead to a high mortality and constitute the first cause of death in Critical Care Units (ICU's). Sepsis is a poorly understood disease and despite life support techniques and the administration of antibiotics, not much more can be done to improve its diagnosis and treatment. The present article has as main objective to discuss the role of neutrophils recruitment in sepsis, dissecting the molecular mechanisms implicated in this complex process and its importance to the pathogenesis of this outstanding cause of death.

## 2. INTRODUCTION

Sepsis remains de first cause of death in Critical Care Units, despite the consistent progress in its diagnosis and treatment during the last decades (1). This can be explained by the fact that, if technology has provided new important tools for the support of critically ill patients, like renal replacement techniques and advances in mechanical ventilation, it has also been implicated as important sources of nosocomial infections. Furthermore, resistance to antimicrobial therapy has been continuously developed by bacteria, as well as immune system evasion strategies. Because of that, sepsis has become a major health problem

worldwide and its pathophysiology, one of the most intriguing enigmas of contemporary medicine.

The importance of a hypothetically explosive proinflammatory response in sepsis began to be questioned about 10 years ago due to the disappointing results obtained by a number of clinical trials who tested agents aimed to control inflammation, such as glucocorticoids, TNFalpha monoclonal antibodies, IL-1 monoclonal antibodies, antibodies anti-endotoxin, antagonists of platelet activating factor (PAF), cycloxigenase and many others (2-9). As a consequence, it was proposed that despite an initial proinflammatory phase, it should be followed by an antagonistic anti-inflammatory response that might provoke significant anergy or severe imunodepression and, in many cases, be patients main cause of morbidity and death (10,11).

Nowadays, it's well accepted that sepsis remains a disease characterized, in its initial phase, by an uncontrolled proinflammatory host response, but it is also clear that the administration of potent anti-inflammatory agents are at least as deleterious as the natural course of the disease. It is believed that for host survival, two points must be matched: the ability to mount a rapid inflammatory response to invading agents and the ability to refrain it and keep it under tight control (12). Both the host response and characteristics of the infecting organism influence the outcome in sepsis (13) and mechanisms of bacterial evasion of the immune system are gaining great attention as important factors for the understanding of the disease process (14).

Following an insult, macrophages and mast cells are activated and release inflammatory mediators, such as histamine, oxygen radicals, platelet activating-factor, cytokines and many other substances, which results in the mobilization of selectins and recruitment of rolling leukocytes to the inflamed tissue. Subsequently, integrins promote firm adhesive interactions to their ligands, expressed on endothelial cells, permiting leykocytes to cross the endothelial barrier.

Thus, neutrophils recruitment is among the first steps, activated by the immune system, to induce inflammation and control infection. Understanding mechanisms of neutrophils recruitment is certainly a crucial area of research for this disease and one of that has been showing the faster evolution. This can be attributed to the discovery and comprehension of the role of many receptors, ligands, secreted molecules and signaling pathways implicated in this complex process.

Neutrophils are key effector cells of the innate immune system. They migrate fast to sites of infection, recognize and engulf microorganisms (phagocytosis) and kill them by the production of reactive oxygen species and antimicrobial and proteolytic granule proteins, delivered to the phagossomes and to extracellular environment (15). Furthermore, neutrophils synthesize chemokines and cytokines, which recruit and regulate the inflammatory process of neutrophils themselves, as well as other cell types, such as T cells (16). Finally, neutrophils are able to induce their own apoptosis, preventing tissue damage by cell lysis and leaking of their deleterious compounds. Neutrophils are terminally differentiated cells with a circulating half-life of about 7 hours. Apoptotic cells are cleared from tissues by resident macrophages.

Although crucial for host defense and repair, neutrophils are also potent inducers of inflammation and immune responses. When emigrating from the bloodstream, neutrophils may injury the vessel wall, inducing thrombosis and edema. Emigrated neutrophils may initiate and sustain tissue damage by the release of diverse mediators, such as oxidants, proteases and cytokines (17).

The present manuscript aims to discuss the most important topics of neutrophils recruitment and the dissection of relevant mechanisms co-related to this subject, establishing a broad and detailed review of its impact in sepsis. Much of that, as will be observed, is based on the analysis of the leukocyte-endothelial cells behavior, when these cell types interact to each other.

## 3. THE LEUKOCYTE-ENDOTHELIAL CELL INTERACTIONS

The interactions among leukocytes and endothelial cells have been implicated in the pathogenesis of a myriad of diseases, as atherosclerosis, gastric ulcers, stroke, malaria and sepsis (18). An intensive effort has been designated to delineate the factors that modulate these interactions. It's already well-known that leukocytes must adhere firmly to vascular endothelial cells to induce the tissue injury and organ dysfunction observed in sepsis. The identification and characterization of a plenty of adhesion glycoproteins has revealed their critical contribution to the adhesion responses elicited by various inflammatory stimuli (19). A brief description of the major cell adhesion molecules and how these interactions are coordinated to ensure an orderly sequence of interactions is here described.

### 3.1. Cell adhesion molecules (CAMs)

Leukocytes recruitment to an inflammatory site begins when these cells slow as they enter in the postcapillary venules, the major site of leukocyte-endothelial cells interactions. The whole process can be divided in the following steps: a) rolling, characterized by low affinity interactions, between leukocytes and venular endothelium; b) adherence, when leukocytes firmly attach on the vessel wall and c) migration (diapedesis), a process of leukocytes transendothelial migration, stimulated by chemotactic signals.

Different families of cell adhesion molecules participate in these steps. The best characterized and probably the most important are the selectins, the integrins and the immunoglobulins supergene family.

#### 3.1.1. Selectins

The selectins are a family of lectin-like molecules with a crucial role in leukocytes rolling. All selectins bind

with low affinity to glycans with terminal components that include alpha2,3-linked sialic acid and alpha1,3-linked fucose, typified by the syalil Lewis x ($sLe^x$) determinant (NeuAcalpha2,3Galbeta1,3[Fuc alpha1,3]GlcNAcbeta1-R). The best characterized ligand is glycoprotein ligand-1 (PSGL-1). PSGL-1 is a transmembrane, homodimeric mucin bearing multiple O-glycans on its serine and threonine residues (20). The selectin family is comprimed of three proteins designated by the prefixes E (endothelial), P (platelet) and L (leukocyte) (21).

L-selectin is broadly constitutively expressed on circulating leukocytes. L-selectin binds to many CAMs expressed on endothelial cells, as P-selectin, E-selectin and GlyCAM.

P-selectin is express on the surface of activated endothelial cells and platelets. This molecule remains stored in Weibel-Palade bodies in endothelial cells and in alpha-granules in platelets. After stimulation, P-selectin is mobilized to the cell surface. While P-selectin participates in leukocyte rolling, platelet-associated P-selectin is involved in leukocytes aggregation with platelets and subsequent thrombi formation (22). Endothelial cells also express P-selectin in response to endotoxin or cytokines. Leukocytes possess many ligands for P-selectin, as L-selectin and P-selectin glycoprotein ligand-1 (PGSL-1).

The requirement for selectins in rolling has been confirmed in mice deficient in L-selectin, E-selectin, P-selectin and PSGL-1 (23-25). Interestingly, leukocytes rolling on activated endothelium can also mediate secondary capture of leukocytes through homotypic interactions (26).

E-selectin is expressed on endothelial cells under transcriptional control. Its syntesis can be induced by proinflammatory cytokines, as IL-1 or TNFalpha, and by endotoxin. Leukocyte ligands for E-selectin include L-selectin and E-selectin ligand.

Beyond its first discovered role in lymphocytes homing and their role in leukocytes recruitment, selectins have also been implicated in a plethora of settings, such hematogenous metastasis of carcinoma cells, effector mechanisms for inflammatory demyelination of axons and implantation of the early mammalian embryo (27).

### 3.1.2. Integrins

Integrins are heterodimeric glycoproteins consisting of alpha and beta subunits. Many integrins are critical factors for leukocyte-endothelial cells firm adhesion. Furthermore, integrins are now known to mediate many other biological functions, as hematopoiesis, immune regulation, hemostasis, embryonic development, cell survival, proliferation and differentiation (28).

The human integrin family now includes at least 19 known alpha-subunits and 8 known beta-subunits. A given alpha-subunit can interact with more than one beta-subunit, resulting in 25 different heterodimers identified to date.

Integrins expression varies from one cell to another (17). An important subfamily, the beta 2 integrins, consists of one of four different alpha chains, named CD11a, CD11b, CD11c and CD11d coupled to a common beta chain, CD18. CD11a/CD18 and CD11b/CD18 are the more well-characterized integrins in leukocytes recruitment. The integrin CD11a/Cd18 (alphaLbeta2, LFA-1) is expressed on the surface of most leukocytes and interacts with ICAM-1 and ICAM-2, leading to firm adhesion on endothelial cells. CD11b/CD18 (alphaMbeta2, Mac-1) and CD11c/CD18, expressed on granulocytes and monocytes, are mobilized to the cell surface on activation of the leukocyte (29). CD11b/CD18 interacts with ICAM-1, but the ligand for CD11c/CD18 remains unknown. Heterodimers of the beta-1 and beta-7 subfamilies also contribute to recruitment of different leukocytes populations.

*In vivo*, neutrophils rolling on selectins use integrins to slow rolling velocities before the cells actually arrest (30). Antibody blocking and intravital imaging of leukocyte recruitment in transgenic mice indicate that alphaLbeta2 (LFA-1, CD11a/CD18) and alphaMbeta2 (Mac-1, CD11b/CD18) are the primary integrins that mediate arrest and transmigration at sites of inflammation (31, 32).

Neutrophils accumulation in inflamed tissues is the balance of the rate of their recruitment and removal. These processes must be tightly regulated to maximize host defense and limit tissue injury. Engagement of Mac-1 to ICAM-1 or fibrinogen signals survival in neutrophils. However, in the presence of pro-apoptotic signals, such as TNFalpha, Mac-1 engagement accelerates apoptosis. Furthermore, Mac-1 dependent phagocytosis of complement-opsonized pathogens triggers neutrophil apoptosis, which is dependent on reactive oxygen species and caspase activation (33).

A number of inflammatory stimulus, like granulocyte-macrophage-colony stimulating factor (GM-CSF) and G-CSF, IL-1, TNFalpha, IL-6, leukotriene B4, C5a and LPS prolong the half-life of circulating neutrophils. Transmigration across the endothelium further delays neutrophils apoptosis.

The importance of beta2-integrins becomes evident in patients with leukocyte adhesion deficiency (LAD-I), a human autossomal recessive disease, leading to a mutation or entire deletion of the CD18 gene. In LAD-II patients, occurs an inappropriate fucosylatation of glycoprotein ligands that are recognized by selectins. These different genotypes share a common clinical profile of recurrent bacterial infections and impaired wound healing (34).

### 3.1.3. Immunoglobulins superfamily

ICAM-1 (intercellular adhesion molecule-1), ICAM-2, VCAM-1 (vascular cell adhesion molecule), platelet endothelial cell adhesion molecule-1 (PECAM-1) and MAd-CAM-1 (mucosal address in cell adhesion

**Table 1.** Adhesion molecules involved in leukocyte-endothelial cell interactions and their most relevant ligands

| Adhesion molecule | Principal ligands |
|---|---|
| *Selectin family* | |
| L-selectin | E-selectin, GlyCAM, CD14, MAdCAM |
| P-selectin | L-selectin, PSGL-1, P-selectin ligand |
| E-selectin | L-selectin, E-selectin ligand |
| *Integrin family* | |
| CD11a/CD18 | ICAM-1, ICAM-2 |
| CD11b/CD18 | ICAM-1 |
| CD11c/CD18 | |
| alpha4beta1 (VLA) | VCAM-1, extra-cellular matrix |
| alpha4beta7 | |
| *Immunoglobulin superfamily* | |
| ICAM-1 | CD11a/CD18, CD11b/CD18, CD43 |
| ICAM-2 | CD11a/CD18 |
| VCAM-1 | alpha4beta1 (VLA), alpha4beta7 |
| PECAM-1 | PECAM-1 |
| MAdCAM-1 | L-selectin, alpha4beta7 |

molecule) are the five members of the immunoglobulins superfamily implicated in leukocytes recruitment.

ICAM-1 is constitutively expressed on endothelial cells, but its expression can be increased upon appropriate cell activation. ICAM-1 binds to CD11a/CD18 and CD11b/CD18 on leukocytes.

ICAM-2 is also constitutively expressed on endothelial cells, but its level is not influenced by the level of cell activation. ICAM-2 binds to CD11a/CD18 and with a lower affinity to ICAM-1.

VCAM-1 binds alpha4beta1 and alpha4beta7 on leukocytes, mediating monocytes and lymphocytes trafficking. Its expression increases upon cytokines stimulation.

PECAM-1, constitutively expressed on platelets, leukocytes and endothelial cells, mediates adhesion of leukocytes and platelets to endothelial cells and the migration of leukocytes through endothelial cells.

MAd-CAM-1 is expressed on high endothelial venules and is involved in lymphocytes homing. It binds to L-selectin and alpha4beta7.

For a list of adhesion molecules involved in leukocyte-endothelial cells interactions and their most relevant ligands see Table 1.

### 3.2. Neutrophils recruitment dynamics

The principal physical factor that influences on neutrophils rolling and adherence is shear stress, the force generated by the blood circulation. Venular cell wall shear stress dictates cell rolling and firm adhesion, because reduction in shear stress facilitates these steps, due to a prolonged contact between neutrophils and endothelial cells, while increases in shear stress tend to oppose them.

Chemical factors are also importantly implicated in neutrophils recruitment to sites of infection. Most of them act are pro-adhesive factors. Examples include histamine, platelet activating-factor, TNFalpha, IL-8 and other chemokines. Remarkable, although chronic stress has immunosuppressive effects (35), acute psychological stress has been shown to induces an increase in leukocytes recruitment in mice into sites of immune activation (36). Moreover, some anti-adhesive factors have also been described, their mode of action still remaining poorly understood. Examples include nitric oxide, PGI2 and adenosine.

Chemokines are a family of small proteins, divided in three groups based on the position of the cysteine residues: CC, CXC and CX3C. About 40 chemokines have already been described (37). They are produced by a variety of cells and have an important role in leukocytes recruitment. Ligation of CXCR by IL-8, for example, activates numerous signaling pathways that culminate in beta2-integrin conformation shift, clustering and adhesion. The secretion of chemokines is stimulated by many factors, notably proinflammatory molecules. Interestingly, many bacteria have the ability to alter the host cytokines synthesis, degrade proinflammatory cytokines and use soluble cytokines receptors as invasion vehicles (38).

Rolling is observed only under low shear stress conditions and adhesive interactions between leukocytes and endothelial cells are mediated predominantly by selectins and their counter-receptors.

Members of the selectin family exhibit sufficiently rapid binding kinetics to support the tethering of unactivated leukocytes on the vessel wall. This process requires the constitutive expression of L-selectin and upregulation of endothelial P- and E-selectin (39).

The initial contact of the leukocyte with the endothelial cell tethers the leukocyte and allows chemoctactic agents, as platelet-activating factor (PAF), IL-8, monocyte chemoattractant protein (MCP-1), macrophage inflammatory protein-1 (MIP-1beta) or growth factors, such as granulocyte/monocyte colony stimulating factor (GM-CSF) and other secreted molecules to induce integrins increased expression and changes in their affinity and adhesive avidity. The activated leukocyte integrins are able, at this moment, to establish firm adhesion to their endothelial ligands, which are members of the immunoglobulin gene superfamily.

The main ligand for the beta2-integrins on inflamed endothelium is ICAM-1. LFA-1 (CD11a/CD18) also binds to ICAM-2 and ICAM-3, but with a lower affinity. Compared to LFA-1, Mac-1 binds to more ligands, including complement fragment C3bi, fibronectin, vitronectin, laminin, collagen, ICAM-1, ICAM-2, albumin, myeloperoxidase, kininogen, elastase, heparin and zymosan (39).

The beta2-integrins Mac-1 and CD11c/CD18 also promote the phagocytosis of microbes, recognizing many pathogens directly or in an opsonin-dependent manner.

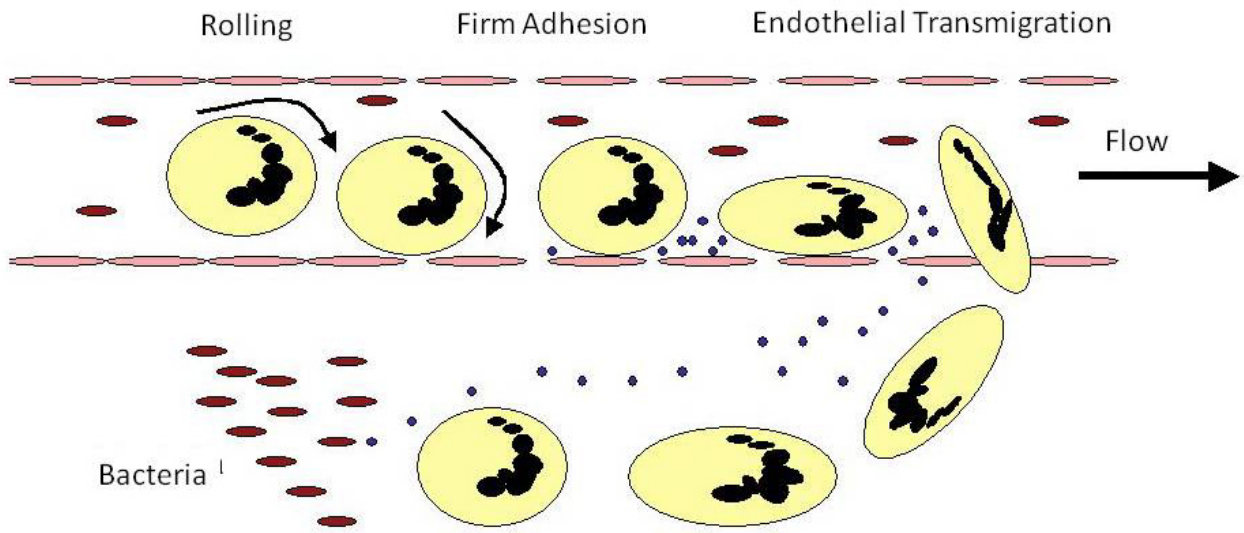


**Figure 1.** Adhesive interactions during leukocyte emigration. Rolling depends mostly of selectins. Firm adhesion and transmigration are dependent of integrins and Ig-like proteins. Cytokines and chemokines are represented as little circles and bacteria as little elipses.

Furthermore, Mac-1 is able to cooperate with a variety of other surface receptors, including Fc receptors, Toll-like receptor 2 and CD14.

After firm adhesion, leukocytes migrate through the endothelial cells surface and intercellular junctions (Figure 1).

However, there are some exceptions to this general model. Tethering and rolling can also occur via 4beta7, alpha4beta1 and alphaLbeta2. Leukocytes interactions to platelets may contribute to recruitment to inflammatory sites. Platelets adherence to damaged vessels wall may recruit leukocytes directly. A number of important pathways are not clearly characterized.

Moreover, selectin-mediated rolling alone is not sufficient to enable cells to firmly adhere but is synergistic with signaling via chemokine ligation in activation of integrins to bind ICAMs and VCAM-1 more efficiently under shear flow. The intracellular cascade associated with activation through L-selectin appears to be mediated through tyrosine phosphorylation of L-selectin's cytoplasmic domain, which activates downstream kinases (Src, Raf/Rak, ERK, p38 and many others), culminating in integrins activation and cell adhesion.

### 3.3. Tool-like receptors (TLRs)

The immune system ability to discriminate between self and foreign pathogens relies; to a great extend, on Toll-like receptors. TLRs are a family of transmembrane proteins with leucin-rich motifs in their extracellular portions. Ten human TLRs have been identified so far and their ligands are predominantly pathogen-associated molecular patterns (PAMPs), conserved microbial molecular patterns (40). TLRs are key effector of the immune response, activating multiple steps of the inflammatory reaction, such as production of proinflammatory cytokines, generation of oxygen reactive species and others. Recently, it has been showed that TLRs are also able to regulate neutrophil recruitment and to delay neutrophil apoptosis (41).

Activation of TLRs by PAMPs facilitates neutrophil recruitment by up-regulation of endothelial adhesion molecules expression through activation of TLRs on endothelial cells themselves or indirectly, via cytokine release from other tissue cells such as macrophages (42). Local generation of chemokines in response to TLR-mediated activation of macrophages, epithelial and endothelial cells enables tight adhesion of neutrophils and localization of these cells to sites of infection. Furthermore, many other inflammatory mediators also present at sites of infection (C5a, some bacterial peptides and others), also regulate neutrophil chemokine receptors in complex patterns (43).

Activation of TLR2 and TLR4 have been associated to prolonged neutrophil survival (44, 45) and to proapoptotic effects in cell types other than the neutrophil (46). Its clear that pathogenic bacteria induce host cells apoptosis through the activation of TLRs to limit the lifespan of cells and control the duration of host defenses.

### 3.4. Immunoglobulins Fc receptors (FcRs)

The antigen-antibody interaction is recognized at the cell surface by a class of glycoproteins, which belong to the immunoglobulins superfamily and are named immunoglobulins Fc

receptors (47, 48). Antibody-FcR interaction activates a number of cell responses, such as antigen presentation, antibody-dependent cellular cytotoxicity, degranulation, endocytosis, phagocytosis, transcytosis and production of proinflammatory mediators. FcRs, moreover, are able to induce inhibitory signaling, participating in the tight control of the inflammatory process and in the maintenance of body homeostasis (49). Different FcRs are expressed in different cells types and the same FcR can induce different responses, depending on the cell type.

Circulating immune complexes are produced continuously in response to infection, trauma and autoimmunity. In particular, IgG-containing immune complexes are implicated in the pathogenesis of systemic lupus erythematosus, rheumatoid arthritis and glomerulonephritis. Evidence that activated complement components may also be required for IC deposition has also been shown. C1q is required for IC deposition to endothelial cells *in vitro* (50), activating C3 to C3b, which facilitates IC binding to cell surfaces. IC deposition initiates events that culminate in leukocytes recruitment and activation. ICs bind do FcRs and activate complement (51). Complement activation may recruit leukocytes through chemokines release or augmenting the expression of adhesion molecules. Heterologous ICs do not induce a systemic inflammatory response (52). Furthermore, using a mouse model of immune-mediated inflammation, it has been shown that vascular permeability promotes IC deposition, but Fc gamaRs are not required for this. ICs do not affect numbers of rolling leukocytes, but significantly slow rolling velocity, which may favor adhesion, by prolonging transit times. Fc gamaRs, despite not required for IC deposition, have shown a critical role in IC-induced slow rolling and adhesion, supporting an important role for leukocyte FcRs in IC-induced leukocytes recruitment (52). IC-induced adhesion and endothelial transmigration (diapedesis) was shown critically dependent on FcgamaRIII. The role of these mechanisms is obscure in sepsis and remains to be investigated.

In neutrophils, integrin signaling is crucial for functions as firm adhesion, cell spreading, chemotaxis, production of reactive oxygen species and the release of various cytokines. This demonstrates that tight control is required over integrin signaling. Recently, it was described that integrin signaling in neutrophils and macrophages proceeds by an immunoreceptor-like mechanism using the ITAM-containg DAP-12 and the FcR gama adaptor proteins to couple integrin signaling ligation to syk activation and downstream signaling events (53). These adaptors are not critical for the CD18-dependent migration of neutrophils, despite being required for other CD18-dependent functions. DAP-12 and FcRgama are not required for adhesion-independent neutrophil responses triggered by cytokines, Toll-like receptors ligands, chemokines or bacterial chemoattractants. These results indicate that ligation of integrins lead to phosphorylation of DAP-12 and FcRgama by members of the Src kinase family, allowing the adaptors to associate with Syk by an ITAM-SH2 interaction, leading to Syk activation and subsequent downstream signaling (53). The receptors associated to DAP-12 and FcRgama and implicated in this kind of response remain unclear.

## 4. FREE RADICAL PRODUCTION, POLY(ADP-RIBOSE)POLYMERASE ACTIVATION AND ORGAN DAMAGE BY NEUTROPHILS

Endothelial cells generate reactive species (RS), including superoxide ($O_2^{\bullet}$), hydrogen peroxide ($H_2O_2$), $NO^{\bullet}$, peroxynitrite ($ONOO^{\bullet}$), hydroxyl radicals ($^{\bullet}OH$), and other radicals (54). More recently, it has become clear that RS such as $O_2^{\bullet}$ and $H_2O_2$ also have several potentially important effects on endothelial function and phenotype and are implicated both in physiological regulation and disease pathophysiology. $O_2^{\bullet}$ reacts rather poorly with itself to produce $H_2O_2$ and $O_2$, this reaction is substantially accelerated by superoxide dismutase (SOD). Hydrogen peroxide ($H_2O_2$), in the presence of reducing metals, goes on to form hydroxyl and other free radicals. In addition, monocytes and neutrophils contain high levels of the enzyme myeloperoxidase that catalyzes formation of the potent oxidant, hypochlorous acid (HOCl). HOCl can react with extracellular amino acids to generate chloramines, which maintain some of the oxidizing potential of HOCl, in spite is not as potent as HOCl (55). $O_2^{\bullet}$ reacts with NO at a significantly faster rate than with SOD, so that when levels of NO are in the high nanomolar range, $NO^{\bullet}$ may outcompete SOD and react with $O_2^{\bullet}$ to generate $ONOO^{\bullet}$, this reaction also resulting in $NO^{\bullet}$ inactivation (54).

The production of superoxide (by activated neutrophils, xanthine oxidase, NADPH oxidases, and other sources) has been previously recognized as an important cytotoxic factor contributing to vascular damage in various pathophysiological conditions. Recent evidence suggests that the reaction of superoxide with $NO^{\bullet}$ yields a toxic oxidant, peroxynitrite, which plays a central role in the pathophysiology of inflammation and oxidant stress (56). Importantly, under certain conditions, such as cellular L-arginine depletion, NOS can produce both superoxide and $NO^{\bullet}$, and the resulting production of peroxynitrite can exert marked autocrine toxicities (56).

Poly(ADP-ribose) polymerase-1 (PARP-1; EC 2.4.2.30) [also known as poly(ADP-ribose) synthetase and poly(ADP-ribose) transferase] is a nuclear enzyme present in eukaryotes (57, 58). The

primary PARP-1 functions as a DNA damage sensor and signaling molecule binding to both single- and double stranded DNA breaks. Upon binding to damaged DNA mainly through the second zinc-finger domain, PARP-1 forms homodimers and catalyzes the cleavage of NAD+ into nicotinamide and ADP-ribose and then uses the latter to synthesize branched nucleic acid-like polymers poly(ADP-ribose) covalently attached to nuclear acceptor proteins (59).

### 4.1. Cell Death

#### 4.1.1. Necrosis

It is important to note that necrosis is not simply another type of cell death; it represents a more severe form of cell demise compared with apoptosis. Leakage of cell content from necrotic cells into the surrounding tissue may contribute to organ injury. Using NMDA- or peroxynitrite-treated neurons, Lipton's and Nicotera's groups elegantly demonstrated that apoptosis and necrosis are at two ends of a continuum in which apoptosis is caused by mild stimuli and necrosis is triggered by severe stimuli (60). Furthermore, it has also been suggested that both ATP and NAD+ are important determinants of the mode of cell death, especially in oxidatively injured cells (61). From these observations, it was plausible to hypothesize that PARP as a NAD+-catabolizing enzyme may serve as a molecular switch between apoptosis and necrosis.

Moderate PARP activation may decrease cellular NAD+ content without being fatal to the cells. Such moderately compromised cellular energetics may cause cell dysfunction. Pharmacological inhibition of PARP, by improving cellular energetics, may rescue cells from this dysfunctional stage and thereby can restore cell function. Examples of such a restorative effect were found in *ex vivo* experiments in endothelial cells producing high levels of endogenous oxidants during diabetes (62, 58) and in intestinal epithelial cells from colitic guts (63).

#### 4.1.2. Apoptosis

During apoptosis, caspase-7 and caspase-3 cleave PARP-1 into two fragments: p89 and p24 (64, 65). These proteases recognize a DEVD motif in the nuclear localization signal of PARP-1 (66), and cleavage at this site separates the DNA binding domain from the catalytic domain, resulting in the inactivation of the enzyme. Cleavage fragments contribute to the suppression of PARP activity because p89 and p24 inhibit homoassociation and DNA binding of intact PARP-1, respectively (67). The existence of this positive feedback loop in caspase-mediated PARP-1 inactivation suggests that blocking PARP-1 activation is vital for the proper function of the apoptotic machinery. Experimental evidence supporting this hypothesis was provided by Herceg and Wang (68), showing that the expression of a caspase-uncleavable, modified version of PARP in TNF-α-treated PARP-1 knockout fibroblasts leads to NAD+ depletion and necrosis. Inhibition of PARP activity by 3-aminobenzamide blocked both NAD+ depletion and cell death.

Recent data indicate that PARP-1 also plays a central role in a caspase-independent apoptosis pathway mediated by apoptosis-inducing factor (AIF) (69). Translocation of AIF from the mitochondria to the nucleus is dependent on PARP activation in neurons and fibroblasts treated with various DNA-damaging stimuli such as MNNG, NMDA, or hydrogen peroxide (69).

Apoptosis is of fundamental importance because it ensures that cells are removed before plasma membrane integrity is lost. Leakage of intracellular molecules may cause the induction of potentially harmful inflammatory responses. On the surface of the macrophage, CD14, CD91, scavenger receptors, and a receptor specific for phosphatidylserine (PS), have been implicated as important mediators of macrophage recognition of apoptotic cells. The only phagocytosis-associated change known to occur on the apoptotic cell surface is the translocation of PS from the inner leaflet to the outer leaflet of the plasma membrane. This event often requires caspase activation and is observed early during apoptosis. Oxidative stress induces poly-ADP ribose polymerase (PARP) activation, which causes rapid depletion of cellular ATP. Oxidative damage can therefore inhibit apoptotic processes and convert cell death to necrosis. From a physiologic perspective this is important since phagocytosis of necrotic cells is not initiated until after membrane integrity is lost. Interestingly, when ATP levels in H2O2 treated B Lymphoma cells are maintained through the inhibition of PARP, the cells undergo extensive apoptosis and PS is externalized to the plasma membrane surface. However, phagocytosis of these cells is very inefficient. Thus, PS externalization does not guarantee efficient phagocytosis of apoptotic cells, and the removal of these cells requires a factor that is sensitive to H2O2 treatment (70).

### 4.2. Proinflammatory Signal

Ischemia-reperfusion as well as proinflammatory cytokines trigger free-radical formation by stimulating xanthine oxidase activity and also by recruiting activated neutrophils, which express NADPH oxidase as well as a variety of other potential cellular sources. Baseline production of NO from constitutive sources may be supplemented by de novo iNOS expression. As a consequence, the oxidants peroxynitrite, hydrogen peroxide, and hydroxyl radical are formed from the interaction of superoxide and NO. Furthermore, under conditions of oxidative stress, NO may be converted to the more toxic nitroxyl anion (NO•). Oxidant stress generates DNA single-strand breaks. DNA strand breaks then activate PARP, which in turn potentiates NF-κB activation and AP-1 expression, resulting in greater

expression of the AP-1- and NF-κB-dependent genes, such as iNOS, ICAM-1, MIP-1α, TNF-α, and C3. Generation of C5a in combination with increased endothelial expression of ICAM-1, recruits a greater number of activated leukocytes to inflammatory foci, producing greater oxidant stress. It is possible that, on a small scale, PARP-mediated necrosis and PARP-mediated proinflammatory gene expression are beneficial or protective processes. For example, NAD+ depletion and cell necrosis may help eliminate "innocent bystander" parenchimal cells having severely damaged DNA (e.g., caused by a nearby occurring neutrophil attack on invading microbes). It is also possible that a low-level, localized inflammatory response may be beneficial in recruiting mononuclear cells to an inflammatory site. For example, invading microorganisms trigger a local neutrophil oxidant burst, and the DNA injury and PARP activation in nearby professional and nonprofessional immune cells triggers proinflammatory cytokine and chemokine production, which recruits additional mononuclear cells to the site of infection to eliminate the invading microorganisms. It is important to note that the only known mammalian cells that do not contain PARP are the neutrophil and eosinophil granulocytes. It is possible that the presence of PARP in these cells is not compatible with the high levels of local oxidant production that these cells frequently generate. However, in many pathophysiological states, a multitude of experimental evidence makes us conclude that the above-described feedback cycles amplify themselves beyond what can be considered desirable or controllable by the body's own defense systems. The cycle is renewed by multiple positive-feedback cycles as the increase in oxidant stress triggers more DNA strand breakage. The proposed cycle of inflammatory activation will be augmented in systems in which PARP-dependent MAP kinase activation and NF-κB translocation contribute significantly to free-radical and oxidant formation and granulocyte recruitment. According to this proposed model, PARP occupies a critical position in a positive-feedback loop of inflammatory injury. NAD+ depletion induced by PARP activation is likely to accelerate this positive-feedback cycle by preventing the energy dependent reduction of oxidized glutathione, the chief intracellular antioxidant and most abundant thiol in eukaryotic cells (71). NAD+ is the precursor for NADP, a cofactor that plays a critical role in bioreductive synthetic pathways and the maintenance of reduced glutathione pools. The depletion of reduced glutathione, as a consequence of intracellular energetic failure or overwhelming oxidant exposure, leaves further oxidant stress unopposed, resulting in greater DNA strand breakage. The various oxidants and free radicals produced in inflammation frequently synergize with each other, with respect to PARP activation (72) as well as other (PARP-independent parallel) oxidant and cytotoxic processes.

**4.3. Circulatory Shock**

Shock is associated with the enhanced formation of oxyradicals and with the expression of iNOS, resulting in the overproduction of NO. NO and superoxide react to form peroxynitrite, and all three species have been implicated in the pathogenesis of cardiovascular dysfunction and multiple organ failure in various forms of systemic inflammation and shock. In isolated cells and tissues, authentic peroxynitrite is capable of mimicking many of the pathophysiological alterations associated with shock (endothelial and epithelial dysfunction, vascular hyporeactivity, and cellular dysfunction), and these alterations are, in part, related to PARP activation (64). In studies in anesthetized rats, the inhibition of PARP with 3-aminobenzamide and nicotinamide reduced the suppression of the vascular contractility of the thoracic aorta *ex vivo* (73). Peroxynitrite production has been suggested to contribute to endothelial injury in circulatory shock. Peroxynitrite can impair the endothelium-dependent relaxations (74). Data demonstrating the protective effects of 3-aminobenzamide against the development of endothelial dysfunction in vascular rings obtained from rats with endotoxic shock (73) suggest that DNA strand breakage and PARP activation occur in endothelial cells during shock and that the subsequent energetic failure reduces the ability of the cells to generate NO in response to acetylcholine-induced activation of the muscarinic receptors on the endothelial membrane. It is possible that this impairment is related to endothelial depletion of NADPH (an essential cofactor of NO synthase) because of PARP overactivation (62, 58).

The role of PARP activation in the pathogenesis of hemorrhagic shock was recently further investigated in a murine model by comparing the response to hemorrhage and resuscitation in wild-type and PARP-deficient mice (75). There was a massive activation of PARP, detected by poly-(ADP-ribose) immunohistochemistry, which localized in the areas of the most severe intestinal injury, i.e., the necrotic epithelial cells at the tip of the intestinal villi, and colocalized with tyrosine nitration, which is an index of peroxynitrite generation (75).

Several recent studies compared the survival times of wild-type and PARPdeficient mice in response to high-dose endotoxin and compared the degree and nature of liver damage in the two experimental groups. In one study, all PARP-deficient animals survived high-dose (20 mg/kg) LPS-mediated shock, which killed 60% of wild-type animals (76). Similar results were obtained by another independent group led by de Murcia (77). Moreover, LPS-induced necrotic liver damage was significantly reduced in the PARP-deficient mice (76). In contrast, when apoptotic liver damage was induced via injection of low concentrations of LPS (30 μg/kg) into D-galactosamine-sensitized mice or

via activation of hepatic cell-death receptors, PARP-deficient animals were not protected (76).

## 5. CONCLUSIONS

Neutrophils comprise approximately two-thirds of peripheral blood leukocytes and participate in different steps of host defense to infection.

Neutrophils recruitment is a critical step of the inflammatory response. The ordered action of 3 selectins and their ligands, at least 2 integrins and 4 or more super-Ig members explicit the importance of this process, protected in such a redundant manner.

Excessive or inappropriate neutrophil activation to infection can result in exacerbated inflammation and severe tissue damage.

Cell adhesion molecules are important potential targets for the treatment of septic patients. The therapeutic intervention on other molecules equally implicated in neutrophil recruitment (such as TLRs and FcRs) and their potential benefit in the treatment of septic patients merits also appropriate investigations.

PARP activation works as an amplifier of inflammatory process increasing adhesion molecule, cytokines and neutrophil activation.

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**Abbreviations:** CAM, Cell adhesion molecules; TLR, Tool-like receptors; FcR, Immunoglobulins Fc receptors; PAF, platelet activating factor; PSGL-1, glycoprotein ligand-1; IL-1β, interleukin-1beta; TNF-α, tumor necrosis factor alfa; ICAM-1, intercellular adhesion molecule-1; ICAM-2, intercellular adhesion molecule-2; GM-CSF, granulocyte-macrophage-colony stimulating factor; G-CSF, granulocyte-colony stimulating factor; C5a; complement fraction 5a; LPS, lipopolyssacharide; VCAM-1, vascular cell adhesion molecule; PECAM-1, platelet endothelial cell adhesion molecule-1; MAd-CAM-1, mucosal address in cell adhesion molecule; IL-8, interleukin-8; MCP-1, monocyte chemoattractant protein; MIP-1beta, macrophage inflammatory protein-1; syk, spleen tyrosine kinase; Raf, protein kinase and functions as an intracellular activator of cell growth; ERK, extracellular signal-related kinase; PAMP, pathogen-associated molecular patterns; FcR, immunoglobulins Fc receptor; C1q, complement fraction 1q; IC, immune complex; C3, complement fraction 3; C3b, complement fraction 3b; ITAM-SH2, Immunoreceptor tyrosine-based activation motif SH domain; RS, reactive species; O2•, superoxide; H2O2, hydrogen peroxide; NO•, nytrosil; ONOO•, peroxynitrite; •OH, hydroxyl radicals; SOD,

superoxide dismutase; HOCl, hypochlorous acid; NOS, nitric oxide synthase; PARP-1, Poly(ADP-ribose) polymerase-1; DNA, deoxyribonucleic acid; NAD+, nucleotide adenosine dinucleotide; ATP, adenosine triphosphate; NMDA, N-methyl-D-aspartate receptors; DEVD, amino acid sequence Asp-Glu-Val-Asp; AIF, apoptosis-inducing factor; PS, phosphatidylserine; NF-κB, nuclear factor – kappa B; AP-1, activator-protein-1; iNOS, inductible nitric oxide synthase.

**Key Words:** Neutrophil, Sepsis, Adhesion, PARP, nitric oxide, Fc receptors, ICAM, VCAM, Review

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