MAEDICA – a Journal of Clinical Medicine
2022; 17(2): 395-403
https://doi.org/10.26574/maedica.2022.17.2.395

MÆDICA - a Journal of Clinical Medicine

REVIEW

Perspectives on the Immune System in Sepsis

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ABSTRACT

Beyond the modifications shown by the biochemistry labs, profound and ample modifications are seen in septic patients at a molecular level stemming from DNA translation and gene expression, manifested as unique profiles of mRNA (messenger), as well as non-coding, functional RNAs: miRNA (micro) and lncRNAs (long non-coding). Counteracting these modifications requires treatment with pleiotropic molecules and/or combination of molecules and opens the possibility of future treatments with arrays of siRNAs and/or specific panels of small molecules tailored for each patient subpopulation.

Keywords: sepsis, immune system, NF-κB, lncRNA, miRNA.

INTRODUCTION

The recent, ongoing SARS-Cov-2 pandemic has brought to the forefront the care of critically ill infectious patients, initially mainly as a respiratory pathology – ARDS – but which has proven to be a multisystemic deterioration with extensive vasculitis affecting mostly every organ, including the lungs, nervous system, liver, biliary tract, pancreas and the gastrointestinal tract, kidneys, heart, and in some of the severely affected patients, multisystem organ failure (MSOF).

Many of the severe manifestations are due to an overactive, imbalanced immune system which produces a "cytokine storm" – an intense discharge of inflammatory molecules produced by activated neutrophils, which is followed by overactivation of the coagulation cascade, with thrombembolism, ischemic tissue changes affecting organ function and ultimately, cellular apoptosis.

In some patients, these modifications are more ample and affect more tissues, especially as the infection is allowed to progress, making this aspect crucial: early, prompt treatment can impede infection progression to the point it overwhelms the immune system, since there are multiple ensuing modifications that simultaneously affect various organs and lead to a very fragile functional state,

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Article received on the 30th of March and accepted for publication on the 8th of June 2022

which is more difficult to normalize *via* external interventions (oxygen, intravenous medication, etc).

Clinical diagnosis and sepsis biomarkers

Sepsis is defined as the simultaneous presence of three elements (1, 2): a) systemic infection (identifiable blood pathogen); b) dysregulated host response, and c) life-threatening organ dysfunction; their presence can be identified with specific clinical signs and symptoms, cellular markers (enzymes released after destruction of cells), cytokines (the immune host response to infection), microbiologic markers (presence of specific bacteria or viruses) and cardiovascular modifications (SEPSIS-3).

Organ dysfunction alone is associated with patient mortality greater than 10% and can be identified with a qSOFA (quick sequential organ failure assessment) score of two or more points; qSOFA allows a rapid warning for sepsis by observing the presence of at least two of the following: 100 mm Hg or less for systolic blood pressure; altered mentation and a respiratory rate of 22/minute or more.

A systemic inflammatory response syndrome (SIRS) requires the presence of two of more of the following five elements: 1) hyper-/hypothermia (>38° C or <36° C); 2) pulse > 90 bpm; 3) respiratory rate >20/min; 4) white blood cells >12,000 or <4000/mm^3 or >10% immature bands; 5) $PaCO_2$ <32 mm Hg (3).

The presence of septic shock necessitates vasopressors to keep mean arterial pressure above 65 mm Hg, or lactate blood level greater than 18 mg/dL without hypovolemia, and is associated with a mortality rate higher than 40%.

Prompt diagnosis and early intervention in sepsis patients is essential, and achieving this goal requires the use of evaluation tools that quantify risks by using specific scores, especially in the emergency departments: qSOFA; SIRS; APACHE II (Acute Physiology and Chronic Health Evaluation II); NEWS (National Early Warning Score, and more recently, REMS (rapid emergency medicine score) (4).

These clinical tools allow an accurate assessment of risk scores for sepsis and prompt therapeutic intervention in patients admitted through emergency departments for in-hospital and seven-day mortality; however, sepsis also occurs in hospitalized patients (10-40% of all sepsis cases in the US); clinical-based scores cannot differentiate between sepsis caused by bacterial *vs* viral infections to guide an efficacious treatment. Blood cultures yield results after too many days when hours matter, and so new diagnostic tools are needed; promising new directions with excellent results are being developed by using mRNAs (5), which will be discussed below.

Sepsis involves dynamic alterations of the immune system, mostly pro-inflammatory in initial phases and anti-inflammatory subsequently, but sometimes markers of both states are detected simultaneously (6, 7); sepsis also entails coagulation modifications, metabolic changes, including hepatic, endocrine, cardiovascular alterations including endothelial dysfunction, both central and autonomic nervous system, and renal alteraions (8, 9). Most organs are affected at a cellular level *via* oxidative stress and ischemic modifications of mitochondria, peroxisomes, Golgi system, lysosomes and cell membranes (10), nuclear transcription of genes and also by the presence of interfering viral material in the cytoplasm. This complexity overlaps underlying individual characteristics such as age, comorbidities including infection source, trauma including surgery, and current medication.

The number and the dynamic modifications of the factors underlying sepsis pathobiology make the use of animal or computer models of sepsis superfluos and the task of finding biomarkers for sepsis staging, prognosis and treatment very challenging (11).

The most frequently used biomarkers for sepsis risk stratification and prognosis include lactate, which was shown to be associated with mortality rates (12); fibrinogen (13); calprotectin (14); macrophage migration inhibitory factor (MIF) (15); copeptin (16); lipopolysaccharide-binding protein (17); the widely used procalcitonin, which is elevated in sepsis patients but also in bacterial infections; and the non-specific inflammation marker C-reactive protein (CRP) (18).

A recent analysis of sepsis biomarkers (19) revealed 258 such molecules, of which 28 were studied in clinical trials enrolling over 300 participants; nine molecules had a better diagnostic value than either/both procalcitonin or CRP, and five of them were identified in adult non-surgical patients: CD64 (20), heparin binding protein (21); decoy receptor 3 (22); Group II phospholipase A2 (PLA2-II) (23); sCD163 (24).

Evaluating the transcriptome, metabolome and proteome in sepsis patients with multimodal assays yields better results for risk stratification and prediction, especially when employed on specific populations such as children (25) and adults (26). This approach may also better differentiate sepsis from non-infectious pathologies like trauma, disseminated intravascular coagulation or pancreatitis.

Pathobiology

The inflammatory response is the most important and challenging aspect in sepsis. It is present not only as contrasting and biphasic pro-inflammatory and anti-inflammatory states, but also with interconnected and simultaneously active molecules and paths. A good exemplification is offered by the natural killer (NK) lymphocytes, which can exert both actions *via* cytokines with pro-inflammatory actions (interferon-gamma IFN-γ, interleukin-1- IL-1, tumor necrosis factor – alpha -TNF-alfa) and anti-inflammatory cytokines such as IL-10, IL-4, transforming growth factor-beta (TGF-β) (27). These pro/anti-inflammatory actions seem to be related to the severity and mortality of sepsis, and also the ensuing lymphopenia and immunoparalysis state (28). Regulatory mechanisms of inflammation involve multiple molecules and pathways which partially overlap, beginning with gene transcription as the essential component, followed by processing of mRNA, translation, phosphorylation, degradation; of these steps, gene transcription and its activation/inhibition are the most important ones (29). Examples of genes undergoing transcription in early stages of inflammation include *Cxcl2, Ptgs2/Cox2, Tnfa,* and *Il1b*. The following genes are expressed in later stages: *Ccl5, Il6, Il12b, Ifnb1, Nos2/INOS, Saa3* and *Marco*. Inflammation pathways include the nuclear factor-κB (NF-κB), mitogen-activated protein kinase (MAPK), and JAK/STAT (29).

NF-κB is one of the most important molecules related to inflammation – when inactive, it forms dimers located in the cytoplasm, where it is bound by IκB proteins. Inflammatory signals such as TNF-α, IL-1β, bacterial LPS, reactive oxygen species and Toll-like receptors (TLRs) cause IκB degradation and translocation of the NF-κB dimers to the nucleus, where they promote specific gene transcription encoding for molecules which regulate cell survival, proliferation, differentiation, and also adhesion molecules, cytokines and various proteins (30, 31).

Due to the complexity and overlapping modulation of the inflammatory pathways, a good strategy for getting a clear snapshot of the inflammatory milieu is to look for actively transcribed genes by analyzing the transcriptome for specific mRNAs.

To achieve this goal, a blood-based diagnosis test was developed, which quantifies 29 host mRNAs and can differentiate infectious *vs* non-infectious pathologies, predict disease severity and 30-day mortality and differentiate bacterial *vs* viral infections (32). To detect the presence of infection, 11 mRNAs are used: the up-regulated genes GNA15, BATF, C3AR1;CEACAM1, ZDHHC19, and C9orf95, and the down-regulated genes RPGRIP1, HLA-DPB1, KIAA1370, TGFB; seven mRNAs are used to distinguish bacterial from viral infections: IFI27, JUP, LAX1, TNIP1, GPAA1, CTSB and HK3; and 11 mRNAs can help predict the 30-day mortality risk: LY86, TST, KCNJ2, HIF1A, SEPP1, C11orf74, CIT, DEFA4, CD163, RGS1, and PER1.

This 29-mRNA test was shown to be significantly better at predicting clinical outcomes in sepsis patients than CRP levels, leukocyte and lymphocyte counts and Charlson comorbidity index and better than IL-6 and APACHE II scores (5); it also has a high accuracy in diagnosing bacterial and viral infections (33).

Perhaps the most consequential recent development in human biology was the discovery of the fact that the majority of the human genome (more than 80% of the three billion nucleotides) has a non-coding functional role (compared to less than 2% of nucleotides, which comprises the known protein-coding genes), and also a majority of single nucleotide polymorphism (SNPs), which are linked to various pathologies *via* genome-wide association studies (GWAS), were not found in protein-coding exons; 88% of them are located in the intronic or intergenic sites (non-coding loci) (34).

Also, there are about two thousand different short RNA sequences with 19-22 nucleotides – micro RNAs (miRNAs) and more than ten thousand long non-coding RNAs (lncRNAs) – which have more than 200 nucleotides, some of which being linked to cellular metabolic pathways related to inflammation and ischemia and playing important roles in the immune response and sepsis (35), as detailed below.

During sepsis, specific miRNAs were shown to inhibit the TLR signaling pathway, modulate production of inflammatory cytokines, regulation of endothelial function and the vascular barrier; in later sepsis stages, they also are involved in apoptosis regulation, immunosuppression, coagulation cascade and organ dysfunction. miRNAs can help with better diagnosing and staging of sepsis, and more prompt and effective treatment (36).

MiRNAs shown to be dysregulated in the peripheral blood of sepsis patients include: miRNA-182, miRNA-486, and miRNA-15a (37); miRNA-146a, miRNA-223, miRNA-16, and miRNA-150 (38); miRNA-125a and miRNA-125b (38). Of these, miRNA-223 plasma levels were shown to correlate with inflammation markers and sepsis severity in multiple studies (38, 40, 41); miRNA-125b has a better prognosis prediction and usefulness for disease management than miRNA-125a (42).

miRNA-146a, miRNA-15a, miRNA-125b, and miRNA-146a prevent NF-κB activation by inhibiting TRAF6 and IRAK (38). A drawback for miRNA-based markers was that the miRNA levels were different in sepsis populations with different characteristics and this must be taken into account in different studies (38).

Long non-coding RNAs (lncRNAs) are present in all human cells and are specific to cells types and organs, and also to pathologies, with lower expression levels compared to mRNA, and also less sequence conservation (43). They are linked to key regulatory mechanisms of inflammation, especially controlling transcription of genes involved in the inflammatory responses, enhancing or suppressing inflammation in both gene- and time-specific ways. They interact with RNA-binding proteins and participate in chromatin remodeling, function as scaffolds, guides, decoys or signals during gene transcription (29).

Besides proteins, lncRNAs can also interact with DNA and other RNA through nucleotide base pairing or RNA folding, which generate structural domains. Indeed, lncRNAs regulate gene expression at all levels: transcription, post-transcription, translation, post-translation and epigenetic (44).

During the inflammatory responses, LncRNAs play essential roles by regulating multiple pathways, modifying gene expression in inflammatory cells involved in proliferation and differentiation (eg, M1/M2 macrophage polarization), including production and release of inflammation-related cytokines.

Furthermore, lncRNAs are dynamically regulated by specific stimuli, so the pattern of lncRNAs can unmask cell exposure to distinct inflammatory signals (45).

Also, lncRNAs are targets in various inflammatory pathways and have modified expression profiles in different cells during inflammation; such differences are seen in viral *vs* bacterial infection, differential TLR activation (TLR4 *vs* TLR2) and also the same antigen (LPS) activating different pathways in different tissues, including cardiomyocites, endothelial cells, renal tubular epithelial cells, and monocytes (46).

TNFα regulates more than 50 pseudogene lncRNAs and hundreds of lncRNAs, responding very selectively to specific cytokines and antigens in a NF-κB-dependent manner. TLRs recognize specific patterns from pathogens and have essential roles in innate and adaptive immunity; many lncRNAs are also regulated in response to specific stimuli. Gp96 Convergent responds only to TNFα. H2-T32/24AS only to TNFα and TLR3 agonists; Cox2 Divergent to pro-inflammation cytokines and TLR1-4 agonists; while HoxA11AS responds to TLR3 agonists and is down regulated by TNFα stimulation (45).

lncRNAs can also act as anti-inflammatory factors, limit/inhibit NF-κB signaling and transcription of pro-inflammatory cytokines, and down-regulate inflammatory pathways. LncRNA-MEG3 inhibits production and actions of inflammatory molecules IL-1β and TNF-α and p65/ NF-κB and also macrophage apoptosis induced by LPS by modifying the levels of Bax/Bcl-2 proteins (47).

Lethe is a 697 bp long pseudogene lncRNA specifically induced by TNFα and IL-1β, which inhibits NF-κB *via* negative feedback; it does so by binding to the NF-κB p65 dimer and preventing its binding at target genes, thus acting as a decoy. Lethe is upregulated by TNFα and IL-1β, but not TLR agonists, indicating its involvement in inflammation, although not in the native immune response; its level decreases with age, a state associated with increased NF-κB activity (43).

Similar to Lethe, NKILA down-regulates NF-κB-driven inflammation, but through a different mechanism: it blocks IκB degradation, preventing NF-κB translocation to the nucleus, (a post-translational modification). Yet, another way of inhibiting NF-κB activity, both basal and

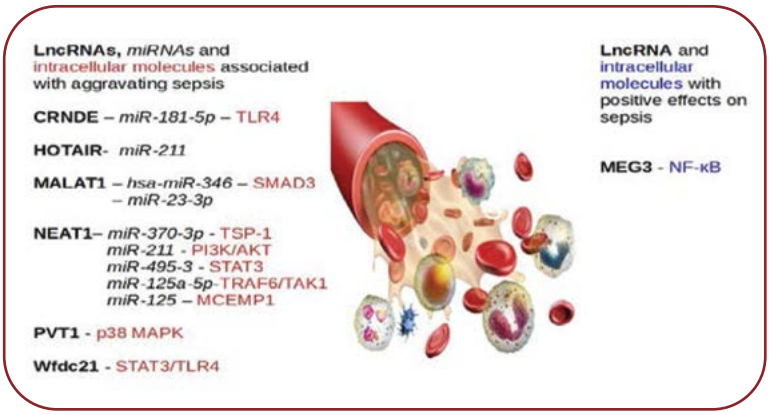

FIGURE 1. Nucleic acids and biomolecules linked to sepsis progression or amelioration

TNF-α-stimulated, is showed by lincRNA-p21, which sequesters p65 mRNA and slows translation of p65 (48).

In septic patients, silencing of lncRNA-5657, lncRNA-MALAT1 and lncRNA-THRIL can protect the lung from inflammation damage; limiting the mitochondrial damage associated with increased levels of lncRNA-NEAT1, lncRNA-HOTAIR and lncRNA-CRNDE can protect from cardiomyopathy, increases in plasma lncRNA-ATP13A4-8z correlated with progression of injury to renal epithelium (43); lncRNA-NEAT1 is correlated with increased liver injury (49) but also with decreased myocardial injury (50); while lncRNA-XIST prevents acute lung injury in sepsis *via* miR-16-5p (51). Figure 1 below shows various lncRNAs, miRNA and molecules acting on immune pathways and linked to aggravation or amelioration of sepsis.

Immune system modifications post sepsis

It was observed that the alterations of lymphocytes mirror the severity of sepsis; moderate sepsis, with mortality below 10%, is generally not followed by a decrease in the number or function of tissue-resident memory T cells (TRMs), the CD103+ cells which are able to detect antigens and activate IFN-γ production (52).

Important modifications were observed in the immune systems of patients who recovered from sepsis and manifested as transient decreases in the number and function of lymphocytes (chronic immunoparalysis), especially of CD8+ T cells, also an altered transcription profile of memory CD8 T cells, which make such patients more vulnerable to reinfections by Listeria monocytogenes (28) and intracellular pathogens (27).

Other examples of post-sepsis immune modifications include a decreased number of CD4 and peripheral – TRM-lymphocytes and dendritic cells, which are essential for the activation of T cells and clearing localized infections. Patients recovered from sepsis also showed a reduced antigen repertoire and cell pool composition and subpopulations, modification of CD4 T cells phenotype and function (53). The mechanism of B and CD4 T cell loss in sepsis seems to be caspase-9-mediated, mitochondria-dependent apoptosis (54).

Recovery of immune protection after lymphopenia parallels an increase in number in the circulatory memory CD8 cells (Tcirc), but not TRM (55), *via* a process named homeostatic proliferation (HP). It was shown that "HP-memory" cells had a similar efficacy against infectious particles as antigen-experienced (conventional) CD8 cells (56). Also, generation of competent HP-memory cells was shown to require three elements: 1) CD8+/CD4(+) T cell interaction; 2) CD40L-CD40 interactions with host cells, and 3) antigen presence and release from endogenous bacteria; absence of these factors yields non-functional HP-memory cells (56). The mechanism of the defective priming of HP-memory cells in the absence of CD4+ T cell interaction involves alteration of CD8+ cells with TNF-related apoptosis-inducing ligand (TRAIL) expression; these altered CD8+ cells will undergo apoptosis (activation-induced cell death) after stimulation by antigens. Interestingly, these defective CD8+ cells regain full function after treatment with IL-2, but not IL-7 nor IL-15 (57). Levels of TRAIL were assessed in patients with sepsis and were shown to increase between days 3-7 (58) but are also inversely associated with sepsis severity and organ dysfunction (58, 59).

However, recent research in bone marrow transplantation showed that such alterations in leukocyte function were reversed in the presence of ascorbate and arginine (60, 61).

Treatment-wise

Clinical trials for medications used in septic patients (eg, inhibitors of TNF-alfa, PAF, IL-1) had poor results when assessed globally (57), which was suspected to be indeed due to patient heterogeneity, so populations were divided into specific phenotypes based on clinical characteristics and biomarkers; in this way it was observed that

specific phenotypes had benefits in morbidity and mortality, which were statistically significant. Treatment with recombinant human thrombomodulin was useful only in sepsis patients who had high fibrinogen degradation products such as FDP and D-dimer levels, and high mortality rates (62); treatment of septic patients with recombinant human activated protein C (rhAPC) also had different results in different patient phenotypes (with different levels of plasminogen activator inhibitor (PAI)-1 and D-dimer) (63).

Another possible explanation for the poor results in therapies with inflammation-related molecules is the high complexity of pathways and the number of molecules involved in inflammation, which makes therapies based on single molecules unlikely "one-size-fits-all" panacea. Recently, this was also seen in COVID-19 treatment, where a combination of monoclonal antibodies showed better outcomes than single molecules.

New molecules with pleiotropic actions in inflammation show good promise. One of those, ethyl pyruvate, has been shown to ameliorate sepsis-induced immunosuppression and protect against secondary infection (64); its main immunomodulatory actions are blocking the activation of NF-κB and ERK (extracellular signal regulated kinase) pathways (65).

Another direction to improve immune function in sepsis is to add to existing treatments better support for the structural integrity and function of the intestinal epithelium with substances known to improve the levels of the tight junction proteins – claudin, zonula occludens (ZO)-1 and occludin. The digestive system is also endowed with the human body's largest immune organ, which is needed to separate the intestinal bacterial flora from the rest of the body and limit the entrance of ingested antigens. The dipeptide alanil-glutamine (AlaGln) was shown to improve the levels of zonula occludens-1 (ZO-1) and claudin-5 when administered intravenously (66). Specifically in sepsis, levels of intestinal epithelial tight junction proteins claudin-3, zonula occludens (ZO)-1 and occludin were improved with administration of emodin (67) and also other rhubarb monomers, including rhein, daucosterol linoleate, 3,8-dihydroxy-1-methyl-anthraquinone-2-carboxylic acid, and 1-O-caffeoyl-2-(4-hydroxy-O-cinnamoyl)-D-glucose) (68).

Intravenous administration of glutathione for amelioration of the intracellular redox state and function of mitochondria is another strategy for improving organ function in sepsis, knowing that glutathione levels are decreased (69).

Finally, because sepsis simultaneously involves multiple systems and organs, we should prioritize treatment with molecules known to have multiple simultaneous positive actions at the immune, cardiovascular, endocrine and metabolic levels; two examples of such molecules include ascorbic acid and the amino-acid L-arginine. Ascorbate and arginine are essential molecules for native and adaptive immunity. Arginine is the precursor for nitric oxide (NO), an essential microbicide used by macrophages and neutrophils, also essential for the activation and maturation of T cells. Ascorbate buffers the oxidative stress resulting from oxidative burst in leukocytes, helps maintain and restore mitochondrial function and thus impede apoptosis, antagonizes the inhibitory effects of 2,3 indoleamindeoxigenase (2,3 IDO) on T cells and together with arginine is essential for T cell maturation and function (70).

Besides its essential role in maintaining the immune function, ascorbate is essential for catecholamine synthesis in the adrenals and thus, it is essential for maintaining the sympathetic tonus in the vascular system, without which hemodynamic collapse ensues. It is also essential for the structural integrity of organs and vasculature because its crucial role in collagen formation, which is actively degraded by matrix metalloproteases during inflammation.

Multiple clinical trials have shown that ascorbate had various benefits in septic patients (decreased mortality, reduced need for vasopressor and mechanical ventilation when administered at 3-10 g/day) (71), also 28-day mortality, number of ICU-free and hospital-free days at 50 mg/kg q 6 h (72, 73) and arginine, respectively (74, 75).

Ascorbate can be regarded as a "poster" molecule, illustrating the fact that pathobiological modifications in various organs and systems in septic patients are taking place concomitantly, even though they are studied separately, and treatment results evaluated in clinical trials tend to offer only partial, precise but segmented answers in such complex pathologies, where more focus should be placed on the pattern of modifications rather than individual alterations. When we look beyond the usual molecules used as dynamic markers in sepsis (procalcitonin, lactate, CRP, etc), we observe profound changes in gene expression,

which drive those modifications, while combined therapeutic interventions are usually beyond the scope of most sponsored clinical trials that usually explore single new drugs. With these in mind, we can say that an important direction for therapeutic progress in complex pathologies with high mortality rates may be represented by individually tailored treatments (eg, with arrays of siRNAs and/or panels of small-molecules), which simultaneously and comprehensively address modifications at the nuclear, cytoplasmic and organelle levels in cell function. ❑

Conflicts of interest: none declared.
Financial support: none declared.

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