Epigenetics as the Driving Force in Long-Term Immunosuppression

Jose Luis Garcia Gimenez[1-3,5],
Nieves Edurne Carbonell[3,6],
Carlos Roma Mateo[1-3,5],
Eva Garcia López[2], Lorena
Palacios[3,6], Lorena Peiro
Chova[3,4], Ester Berenguer[2,5],
Carla Giménez Garzo[2,3,5],
Federico V Pallardó[1-3,5], and
Jose Blanquer[3,6]

1   Center for Biomedical Network Research
    on Rare Diseases (CIBERER), Institute of
    Health Carlos III, Valencia, Spain
2   Department of Physiology, Faculty of
    Medicine and Dentistry, University of
    Valencia, Valencia, Spain
3   INCLIVA Biomedical Research Institute,
    Valencia, Spain
4   Biobanco INCLIVA, INCLIVA Biomedical
    Research Institute, Valencia, Spain
5   Epigenetics Research Platform, CIBERER-
    UV, Valencia, Spain
6   Department of Critical Care, Clinical
    University Hospital of Valencia, Valencia,
    Spain

Abstract

Epigenetics is an emerging frontier of biology, with the potential for deciphering the intricate molecular and transcriptional cellular programs, therefore contributing to explain the pathological evolution of sepsis, one of the most elusive syndromes in medicine.

The evolution of sepsis depends not only on the pathogen which originated the infection but also on the genetic and epigenetic background of the host. Short-term mortality of sepsis and septic shock is high, being considered a public health concern worldwide. Immunosuppression is the predominant driving force for morbidity and mortality in late deaths and long-term deaths of survivors from a sepsis episode. In this regard, apoptosis of immune cells and complex epigenetic reprogramming in immune and progenitor cells may contribute to the immunoparalysis observed in post-septic patients, who are prone to the apparition of new, opportunistic infections.

Here, we review the literature and expose the most relevant results which explain the epigenetic programs contributing to the progression of sepsis. Furthermore, we revisit the role of circulating histones in the pathogenesis of sepsis and septic shock and finally we discuss about the therapeutic potential of epigenetic drugs in the treatment of sepsis.

Introduction

Sepsis is defined as the host inflammatory response to severe, life-threatening infection, with the presence of biochemical abnormalities and organ dysfunction. It is a major healthcare problem, affecting millions of people around the world every year. Its incidence is increasing owing to the ageing population, immuno-senescence and the resulting impaired immunity. Moreover, it is the most frequent cause of mortality in most intensive care units (ICUs), especially if not recognized and treated promptly [1]. Despite its worldwide importance and being considered a public health concern, accounting for more than $20 billion (5.2%) of total US hospital costs in 2011 [2] public awareness of sepsis is poor.

The short-term mortality of sepsis is 10-40% and even higher for septic shock (30-60%). Most clinical studies examining patients with sepsis have used 28 day mortality as a clinical end point, and although only a few studies on long-term mortality exist, they indicate that long-term mortality is also increased among patients who had survived the acute episode of severe sepsis

Corresponding author:
Jose Luis Garcia Gimenez

✉ j.luis.garcia@uv.es

Departament de Fisiologia, Facultat de Medicina i Odontologia, Universitat de València, CIBERER, Biomedical Network Research Center for Rare Diseases, INCLIVA Biomedical Research Institute, Avda Blasco Ibanez No 15-46010, Valencia, Spain.

Tel: +34963864646

or septic shock [3]. A large cohort study performed by Quartin et al. [4] found that survivors of sepsis had an increased risk of death that persisted for up to 5 years following the initial

septic event, even after accounting for their underlying medical comorbidities. Recent data demonstrate that survivors have poor quality of life, frequently develop cognitive and functional disability, and require substantial ongoing acute and long term care [5]. In addition, higher long term mortality has been reported in patients with community-acquired severe sepsis or septic shock, as compared to individuals of similar age, sex and comorbidities [6]. Possible causes for the long-term impairments observed include hypoperfusion, toxins, maximal stress response (cytokines), exposure to treatments (such as steroids), immobility, development of complications such as acute lung injury or combinations of these and other factors.

The current paradigm regarding the host immune response to sepsis is under debate [7-11]. Some theories are being considered in order to explain the nefarious consequences of sepsis on the survivors. The most recent and accepted theory states that sepsis consists on an initial hyper-inflammatory phase which can produce "early" deaths during the first 24 h-48 h (e.g. in the toxic shock). Two other theories, based on the immune response, try to explain the morbi-mortality and "late deaths" induced by sepsis few days after hyper-inflammatory phase. The first relies on a persistent activation of innate immunity that would lead to hyper-inflammation, which in turn may cause multiple organ dysfunctions [12]. The second theory - and most widely supported - proposes that after an initial phase of hyper-inflammatory response, a failure or exhaustion of adaptive immunity (T-lymphocytes) occurs, that produces immuno-paralysis or immunosuppression **(Figure 1)**. This phase of immunosuppression produces late deaths due to the exhausted immune response and the apparition of new opportunistic or recurrent infections [8]. The hypothesis that patients enter in a phase of "immune paralysis or immunosuppression" after sepsis or septic shock episodes come from the clinical observation that a considerable proportion of survivors developed both viral reactivation (commonly herpes simplex virus and cytomegalo virus) and secondary nosocomial infections by *Candida* sp., *Acinetobacter* sp., *Stenotrophomonas* sp. [7, 13], etc. Pneumonia also complicates the disease in the late phase of immunosuppression in 10-30% of patients in ICUs, who are treated with mechanical ventilation [14]. An episode of sepsis or septic shock also seems to raise morbidity and mortality in a late stage in successive years producing "long-term" deaths. However, confirmatory studies demonstrating the clinical significance of the "long-term" phase in sepsis are
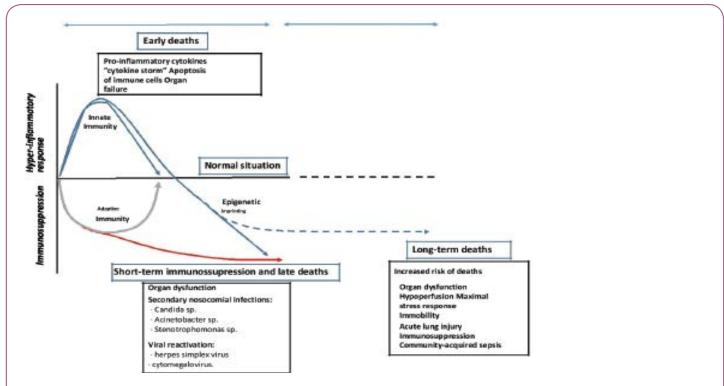

Figure 1 Proposed model for immune responses in sepsis, late immunosuppression and long-term immunosuppression based in the theory of Hotchkiss et al. [8].
Pro and anti-inflammatory immune response occurs steeply during sepsis. Cells of the innate immune system release high levels of pro-inflammatory cytokines that drive the "cytokine storm", and induce apoptosis of immune cells during the first days of sepsis that can produce early deaths. Most patients recover innate and adaptive immunity and survive the infection. However, if sepsis persists, the failure of both the innate and the adaptive immune systems leads patients to enter in an immunosuppressive state that causes the development of nosocomial infections or virus reactivation. Furthermore, survivors may suffer of long-term immunosuppression. In that case, an epigenetic reprogramming in progenitor cells may occur in immune tissues, like spleen and thymus, during sepsis. This epigenetic reprogramming may allow progenitor cells to perpetuate the epigenetic marks into differentiated cells, compromising the immune response and facilitating community-acquired secondary infections (model based in the theory by Hotchkiss et al. [8].

largely missing. The few studies available suggest that the "long-term" phase of sepsis is associated with significant re-increase of positive blood culture results, especially opportunistic bacteria and fungi [15], and patients who survived sepsis remained the strongest predictor of recurrent infections post-discharge, re-hospitalizations for infection, and post-discharge mortality [16].

At the present time, it is completely unclear whether patients who acquire sepsis are simply inherently at risk for infection complications, or whether sepsis imposes additional susceptibility for subsequent infections. It is accepted that, during sepsis, increased apoptosis causes the depletion of immune cells, including both innate immune cells (dendritic cells, follicular dendritic cells, natural killer cells, and myeloid-derived suppressor cells) and adaptive immune cells (CD4+ T and CD8+ T cells and B cells). Among others, circulating histones mediate the apoptosis of these immune cells, therefore compromising the immune defenses and contributing to immunosuppression after septic shock, hence producing late deaths. Furthermore, the efficacy of the immune response requires coordinated mechanisms involved in the activation of a large number of genes participating in the innate immune system [17], which in turn depends on the accessibility of the transcription factors to the chromatin, histone post-translational modifications, and DNA methylation. In this regard, active genes in immune cells are associated with nucleosomes enriched for open-chromatin marks such as trimethylated histone H3 at Lys 4 (H3K4me3) and acetylated histones H3 and H4 [18]. In contrast, H3K9me2 and H3K27me3 are involved in gene silencing in immune cells [18, 19]. Furthermore, the high stress imparted on the immune system during a sepsis or septic shock episode affects seriously the function of myeloid cells, which has important effects on epigenetic signatures in these cells. Therefore, differentiation and maturation of immune cells, together with key genetic programs, are severely affected, thus producing fatal consequences in the innate immune response and increasing the risk of survivors for long-term deaths [20].

As we review in this work, intricate epigenetic mechanisms mediate immune responses during sepsis, demonstrating that epigenetics is an important driving force during immunosuppression, offering a more complex and plausible explanation to the compromised immune response after a septic episode, producing "late deaths" and "long-term deaths".

Sepsis, a Severe Inflammatory Response and Life-Threatening Infection with Organ Dysfunction

The first definitions proposed in 1991 for the sepsis syndrome were based on the presence of presumed infection and at least two out of four Systemic Inflammatory Response Syndrome (SIRS) criteria. Moreover, severe sepsis was considered when acute organ dysfunction secondary to documented or suspected infection concurred; and septic shock (SS), if there was hemodynamic instability that required vasoactive support despite adequate fluid resuscitation [21]. A 2001 Task Force, recognizing limitations within these initial definitions, included an expanded list of both clinical and laboratory abnormalities, yet without offering any alternative due to the absence of new evidence [22]. However, in the last fifteen years increased knowledge of the etiology and pathobiology of sepsis, such as changes in organ function, morphology, cell biology, biochemistry, immunology, and growing evidence of poor clinical and epidemiological utility of previous definitions, have highlighted the need for new terms.

In this regard, a new Task Force with expertise in sepsis was recently convened by the Society of Critical Care Medicine and the European Society of Intensive Care Medicine [23-25]. They considered some limitations from previous definitions, as an excessive focus on inflammation, an inadequate specificity and sensitivity of the SIRS, and the misleading model that sepsis follows a continuum, through severe sepsis to shock. It was concluded that the term "severe sepsis" was redundant, and recommended the elimination of the terms "sepsis syndrome", "septicemia" and "severe sepsis". Besides, the Task sought to differentiate sepsis from uncomplicated infection. Finally, they defined sepsis as life-threatening organ dysfunction caused by a dysregulated host response to infection, and SS was defined as a subset of sepsis in which particularly profound circulatory, cellular, and metabolic abnormalities were associated with a greater risk of hospital mortality than with sepsis alone (>40% *vs.* >10%). The organ dysfunction in sepsis may occur at different time in different patients (before, during and after infection is recognized), establishing a time window including both short (6 h) and long (72 h) around the onset of infection. New definitions were designated SEPSIS-3, emphasizing the need for future iterations **(Table 1)**.

Like for many syndromes, there is no gold standard diagnostic test for sepsis. Thus, clinical criteria were evaluated and established by the Task [23]. They recommended the use of an acute change of SOFA (Sequential Organ Failure Assessment) score of two points or more, consequent to infection, as criteria for sepsis in the ICU setting; and the use of qSOFA (quick evaluation of three parameters: systolic blood pressure <=100 mm Hg, respiratory rate >=22/min, and alteration in mental status) in the non-ICU settings to consider the possibility of sepsis. These scores were chosen based on their significantly higher predictive validity for in-hospital mortality in the different settings. On the other hand, SS could be clinically identified by a vasopressor requirement to maintain a mean arterial pressure of 65 mm Hg or greater, and by the presence of serum lactate level greater than 2 mmol/L in the absence of hypovolemia [24]. The adjusted odds ratio (OR) for hospital mortality increases linearly with increasing of serum lactate level, positioning it as a proxy for a cellular metabolic abnormality. Although not specific for sepsis, it should face validity given the lack of a superior available alternative.

The epidemiologic strengths of the new consensus definitions SEPSIS-3 are counteracted by weakness in their ability to be used in the treatment of individual patients. Given the heterogeneity of molecular and cellular responses associated with septic condition, development of new biomarkers which allow identification of specific patterns may be helpful for categorization of this kind of critically ill patients, and could contribute to future SEPSIS-4

Table 1 Changes in consensus definitions of sepsis, severe sepsis and septic shock.

Terms	Bone et al. [21]	Levy et al. [22]	Singer et al. [25]
Sepsis	The systemic inflammatory response to infection manifested by two or more of the criteria previously defined for SIRS: (1) temperature >38°C or <36°C (2) heart rate >90 beats per min (3) Respiratory rate >20 breaths per minute or $PaCO_2$ <32 mm Hg and (4) White blood cell count >12,000/cu mm, o< 4,000/cu mm or >10% in mature forms.	Clinical syndrome defined by the presence of both infection and a systemic inflammatory response.	Syndrome shaped by pathogen factors and host factors leading to a life-threatening organ dysfunction caused by a dysregulated multifaceted host response to infection.
Severe sepsis	Sepsis associated with organ dysfunction, hypo-perfusion, or hypotension. This may include but are not limited to, lactic acidosis, oliguria, or acute alteration of mental status.	Sepsis complicated by organ dysfunction	Recommend limitation of use of this term.
Septic shock	Sepsis induced hypotension despite adequate fluid resuscitation along with the presence of perfusion abnormalities that may include but are not limited to, lactic acidosis, oliguria, or acute alteration in mental status. Patients who are receiving inotropic or vasopressor agents may not be hypotensive at the time that perfusion abnormalities are measured.	State of acute circulatory failure characterized by persistent arterial hypotension despite adequate volume resuscitation, in the absence of other cause of hypotension.	Subset of sepsis in which underlying circulatory and cellular/metabolic abnormalities are profound enough to substantially increase mortality. Persisting hypotension requiring vasopressors to maintain MAP ≥ 65 mm Hg and having a serum lactate level >2 mmol/L (18 mg/dL) despite adequate volume resuscitation.

definitions, establishment of new treatment targets, to design of new therapeutic approaches and to predict future outcomes in survivors.

In addition, sepsis is a syndrome that encompasses a still-uncertain physiopathology. It is characterized by a complex systemic biphasic immunological response to a microbial pathogen. The presence of pathogens in the bloodstream causes an innate immune response characterized by the stimulation of monocytes and neutrophils and release of pro-inflammatory cytokines like TNFα, and a counter regulatory anti-inflammatory reaction (i.e., mediated by IL-10) as negative feedback on the inflammation. The intensity of the initial inflammatory response varies in individual patients depending on multiple factors, including pathogen load and virulence, patient co-morbidities and host genetic factors. Simultaneously, it results in an activation of a medley of different immune pathways (major expression of tissue factors, which activates coagulation, and an increase in nitric oxide synthesis, which induces vasodilatation), and usually determines the destruction of the pathogen. Toll-like receptors (a group of transmembrane proteins) act as innate immune system sensors through the recognition of highly conserved components of a variety of microorganisms. Under some circumstances, many of these beneficial components of inflammation can be deleterious, causing cell and tissue damage and hence multiple organ failure [26]. It can promptly occur due to a hyperinflammatory 'cytokine storm' response with fever, refractory shock, acidosis and hypercatabolism. However most patients have a restoration of innate and adaptive immunity and survive the infection. Nowadays, understanding of the immune response to sepsis is controversial. Although immunosuppression, as a marked compensatory anti-inflammatory response, seems to justify late-deaths, less is known about the etiology of long-term

deaths, being epigenetics hypothesized as the main conductor.

Despite critically ill patients receive care for a large variety of disease states, the leading causes of death in the ICU are multi-organ failure, cardiovascular failure, and sepsis [27]. Multi-organ failure has a mortality rate of up to 65% when more than one organ system fails [28] new-onset renal failure has a mortality rate of up to 61%, and severe respiratory failure has a mortality rate ranging from 20% to 50%. Sepsis, the second leading cause of death in non-coronary ICUs, has a mortality rate of up to 45%. In patients who are diagnosed with sepsis, up to 51% develop acute renal failure, and up to 20% have acute respiratory failure requiring mechanical ventilatory support. Importantly, septic ICU patients have significantly higher mortality when compared to non-septic critically ill patients [55% *vs.* 19.3%, OR=2.21 (1.65-2.97)]. Acute tubular necrosis and oliguria in septic patients, as well as cardiovascular failure (by SOFA score) in non-septic, have been identified as independent risk factors for mortality [26]. Overall, mortality rates in patients admitted to adult ICUs average 10% to 29%, depending on age and severity of illness. The mortality rate for patients who have been admitted to the ICU is greater for the following 10 years after they leave the ICU compared to patients of the same age who have never been admitted to the ICU [29]. These intriguing data suggest that some kind of "fingerprint" may occur in ICU patients after a critical health situation, which may depend on the severity of the pathology.

Epigenetic Changes Can Explain the Molecular Causes of Long-Term Immunosuppression

Epigenetics is a rapidly growing field of research which studies

the heritable changes (in the progeny cells or of individuals) in the gene activity and expression that do not involve changes in the nucleotide sequence, and also stable, long-term alterations in the transcription of genes that are not necessarily heritable [30]. Epigenetic gene regulation refers to how a specific structural and chemical configuration of chromatin (mediated mainly by post-translational modifications in histones, PTMs) translates into a defined outcome on its transcriptional status.

Overwhelming inflammation reactions in response to microorganisms exposure and microbial products are conducted by the innate immune system cells (i.e., monocytes and neutrophils), which release high levels of pro-inflammatory cytokines and can produce early deaths in a septic process. Most patients survive to this critical scenario and can restore their innate and adaptive immunity. However, in some cases in which sepsis persists, patients can enter in a process called immunosuppression, immuno-paralysis or post-septic immunosuppression [8], which makes patients more susceptible to infections because hematopoietic cells result to be hypo-responsive to stimuli.

Epigenetics, and especially chromatin remodeling, might act as driving forces involved in the short-term and long-term immunosuppression observed after sepsis episodes. In the phenomena of immunosuppression, several observations reinforce this hypothesis: 1) Early findings demonstrate lower levels of H3K9me2 at inflammatory gene promoters in innate immune cells, as compared with non-immune cells [31] and a rapid demethylation of H3K9me2 occurs at promoters of these genes after lipopolysaccharide (LPS) challenge [32] 2) Repetitive or sustained LPS challenge produces TNF- and IL1-repression - mediated after the recruitment of repressive HMT G9a, heterochromatin protein 1 (HP1) and DNMT3A/B - and a subsequent increase of H3K9me2 and DNA hypermethylation at the promoters of inflammatory genes [33] 3) Epigenetic mechanisms participate in the regulation of key specific genes and also in the maturation, development and regulation of the adaptive immune cells.

Sepsis and the Immune System: Epigenetic Regulation of the Innate and Adaptive Immune Cells

Importantly, the mortality rate for patients who have been admitted to the ICU is greater for the following 10 years after they leave the ICU compared to patients of the same age who have never been admitted to the ICU [29]. In those cases, immunosuppression due to defective innate and adaptive immune responses underlies this late effect. Expression arrays from pediatric patients who died of sepsis also showed altered gene expression profiles indicating immunosuppression [34]. Furthermore, gene expression studies performed in children with septic shock showed that genes participating in the modulation of the innate immunity were upregulated, whereas transcription of genes participating in the adaptive immunity were downregulated [34], confirming a compensatory anti-inflammatory response underlying the process of immunosuppression. Regarding these

results, it should be clarified that the levels of epigenetic changes that occur in hematopoietic cells make progenitors in the bone marrow inefficient to restore the immune system, and therefore affect the ability of the host to initiate an immune response throughout the expression of key genes.

It is known that apoptosis of immune cells may also contribute to immuno-paralysis. In this regard, immune cells such as CD4+ and CD8+ T cells, B cells, and dendritic cells showed high apoptosis with serious consequences for the immunity of the patients who survived [8], a phenomenon observed in all age groups during sepsis. However, other immune cells such as neutrophils (in the innate immune system) and T regulatory (TReg) cells, are resistant to apoptosis. In fact, TReg and myeloid derived suppressor cells (MDSCs), which are considered immunosuppressive cells, are increased in sepsis [35-37].

Beyond the apoptosis of immune cells, the drastic changes observed in the expression of genes involved in the innate and adaptive responses suggest the intervention of epigenetic reprograming at their promoter regions, which could condition the response of the immune system. Proof of that premise is that DNA methylation regulates gene expression programs of related pathways which establish the cellular identity of the immune cells in the immune system [38]. On the other hand, immune cells harvested from the spleen or lungs of patients with sepsis showed markedly decreased expression of anti- and pro-inflammatory genes [39], which may respond to intricate epigenetic reprogramming in immune cells and contribute to the apparition of new opportunistic infections [13-15].

As we describe in this section, sepsis has an important impact in the function of all types of immune cells in both innate and adaptive immune systems. Here, we summarize some of the consequences of sepsis on the immune cells and the possible role of epigenetics: In the innate immune cells **(Table 2)**.

Neutrophils

Neutrophils are essential cells which participate in the early recognition and control of invading pathogens [40], and their maturation and function seems to be disrupted during sepsis. Although neutrophils do not produce large amounts of cytokines, they can produce high levels of IL-10 during sepsis, which is considered an immunosuppressive cytokine [41]. Interestingly, the gene for IL-10 has been described to be regulated by DNA methylation and recruitment of Stat3, Stat4, and histone acetyl transferase p300 at IL10 promoter in autoimmune disease [42]. Chromatin remodeling has also been demonstrated to occur in the expression of elastase [43], a protease produced in neutrophils which acts against gram-negative bacteria [44]. Other key process mediated by neutrophils during sepsis is the NETosis or NET (nuclear extracellular traps) released after microbial and fungal infections [45-47]. During NETosis histone deamination and citrullination occur rapidly, a process which is mediated by the peptidylarginine deiminase 4 (PAD4). This event facilitates the release of histones from cells in the form of chromatin traps or free histones [48-50], as we describe in the next section.

Table 2 Epigenetic alterations in innate immune cells.

Immune cells	Function	Epigenetic alteration	Consequence
Neutrophils	Early recognition and control of pathogens.	Hypomethylation of IL-10 Chromatin remodeling in elastase gene H3 citrullination and NET formation	Anti-inflammatory gene expression programs. High levels of IL-10 during sepsis NETosis and release of free histones to the blood stream.
MSDCs	Accumulate in lymphoid organs after infection. Can block T cell function and promote TReg	HDAC6 and HDAC11	Regulates myeloid differentiation and expansion
DCs	Antigen-presenting cells.	Increase of H3K27me3 and decrease of H3K4me at IL-12 gene after sepsis. Changes in the H3S10p, H3K14ac, and H4K9ac at the promoter of IL-8 after bacterial LPS or flagellin	Low levels of IL-12 after sepsis
Monocytes	Multiple roles in the immune infection	vChromatin remodeling at the promoters of pro-inflammatory and anti-inflammatory genes After LPS stimulation, increased levels of H3K9me2 in pro-inflammatory genes after sepsis	Decreased levels of TNFα, L-1β, IL-1α, IL-6, and IL-12 and increased levels of IL-1RA and IL-10 Down regulation of HLA-DR
Macrophages	Phagocytosis of pathogens, activation and proliferation of T- and B-lymphocytes	After LPS stimulation increased expression of KDM6b and demethylation of H3K27me3	Increased expression of IL-10, Arginase-1, Ym1 and IL-1Rn

Myeloid derived suppressor cells

Myeloid derived suppressor cells (MSDCs) are very fast proliferative cells which accumulate in lymphoid organs after infection. There is not a consensus for the function of these cells in humans. However, MSDCs can either enhance or attenuate the SIRS depending on the phase in which they act [7]. For example, MSDSCs can block specific T cell function [35] and promote de novo development of TRegs [51]. It has been shown that histone deacetylase inhibitors increase MSDCs generation and expansion [51]. Recent studies have demonstrated the role of epigenetic modulators in the function of MSDCs. It has been proposed that the transcription factor Stat3 regulates MSDCs expansion by enhancing the differentiation of immature myeloid cells [52]. Furthermore, the role of HDAC6 in the Stat3 activation has also been documented [53]. More recently, Sahakian et al. [54] have demonstrated the role of HDAC11 in myeloid differentiation and MSDC expansion in HDAC11-KO mice.

Dendritic cells

Dendritic cells (DCs) are highly specialized and versatile antigen-presenting cells that provide a critical link between the innate and adaptive immune responses. DNA methylation has been described to play an important role during differentiation and maturation of DCs [55]. Besides the increase of apoptosis of these cells in patients with sepsis who died [56], DCs showed a reduced capacity to produce cytokines such as IL-12 after sepsis. It is known that IL-12 expression depends on chromatin remodeling processes at its promoter by reduction of the repressive mark H3K27me3 and increase of the activating mark H3K4me. In mice models of sepsis, DCs accumulated in lungs after post-septic process, and DCs exhibited increased recruitment of chromatin silencing remodelers instead of chromatin activators at the IL-12 promoter [57]. In addition, DCs have lower expression of HLA-DR and secrete high levels of IL-10 [58, 59]. Finally, changes in the phosphorylation and acetylation of histones (H3S10p, H3K14ac, and H4K9ac) have been found at the promoter of the pro-inflammatory gene IL-8, after bacterial LPS or flagellin from Legionella pneumophila stimulation in dendritic cells [60].

Monocytes and macrophages

Monocytes are produced in the bone marrow and have multiple roles in the immune infection. They move quickly to the sites of inflammation and infection and then divide or differentiate into macrophages and dendritic cells. It has been demonstrated that lipopolysaccharides (LPS) induce chromatin remodeling [61] at the promoters of pro-inflammatory cytokines [62] in monocytes, reducing the capacity of monocytes to release TNFα, IL-1α, IL-6, and IL-12 and increasing the ability to produce anti-inflammatory cytokines IL-1 receptor antagonist (IL-1RA) and IL-10 [63, 64]. This process seems to be sequential. During primary responses to gram-negative bacteria or LPS, the host produces high amounts of IL-1β and TNFα to induce inflammation and to kill the microorganism. However, after this primary response a heterochromatinization mediated by H3K9me2 at the promoters of these genes has been described in monocytic cell lines from septic patients [65]. Other consequence of endotoxin tolerance on monocytes and macrophages is a downregulation of the expression of HLA-DR [66], which is a mark of monocyte unresponsiveness against infection and a risk factor of nosocomial infections and poor outcome [36, 67].

Macrophages have a defensive role through their ability to carry on phagocytosis of pathogens. They are essential in the activation and proliferation of T- and B-lymphocytes by antigens. In macrophages, LPS stimulation increases the expression of the histone demethylase KDM6B (JMJD3), which demethylates H3K27me3 at the promoters of the anti-inflammatory IL-10, Arginase-1, Ym1 and IL-1Rn [19] therefore increasing the expression of these factors.

Sepsis and septic shock also produce several effects, which have serious consequences during the post-septic period. Among others, reduced lymphocyte proliferation, increased apoptosis

Table 3 Epigenetic alterations in adaptive immune cells.

Immune cells	Function	Epigenetic alteration	Consequence
T-lymphocytes	Control of the adaptive immune response	Heterochromatinization of IFNG and GATA3 genes	Low levels of IFNγ and affected function of TH1 and TH2 cells
TReg	Inhibits the function of monocytes and neutrophils	Unmethylated Foxp3 in TReg cells (FoxP3-TSDR)	Limits the hyperinflammatory response, better outcome in mice with sepsis
B- lymphocytes	Presents antigens to T lymphocyte, and differentite into antibody producing cells	miR-17-92 cluster, miR-124 and miR-181a participate in B cell proliferation and apoptosis by targeting Bim and Pten transcripts miR-34a blocks the B cell development	Aberrant antibody responses to foreign antigens

and cell death of CD4+ T cells and T cell precursors have been found in several tissues during the acute phase of sepsis. In the adaptive immune cells **(Table 3)**.

T-lymphocytes

T cells or T-lymphocytes are a type of white blood cells essential for human immunity controlling the adaptive immune response. Sepsis and septic shock also produce several effects, which have serious consequences during the post-septic period. Among others, reduced lymphocyte proliferation, increased apoptosis and cell death of CD4+ T cells and T cell precursors have been found in several tissues during the acute phase of sepsis. One of the mediators of the immunosuppression is IL-10. The production of this anti-inflammatory cytokine activates mature CD4+ TH2 cells and inhibits CD4+ TH1 cells. Early studies suggested that subpopulations of CD4+ TH2 and CD4+ TH1 cells are decreased after sepsis, compromising the effector responses [68]. In addition, heterochromatin marks in histones are thought to mediate the repression of IFNG and GATA3 genes, therefore affecting the function of TH1 and TH2 cells [69]. On the other hand, in mice models, CD4γ T cells programmed to become CD4+ TH1 effector cells are decreased in post-septic naive CD4+ T cells. This effect is consequence of the increase in the H3K27me3 mark at the promoter region of IFNγ [69].

Regulatory T cells

Regulatory T cells (TReg). CD4+ T cells are able to increase the differentiation of TReg, which participate in the immunosuppression process by inhibiting both monocyte and neutrophil function [70], although their role during the hyperinflammatory and the immunosuppression phases is still unclear and sometimes contradictory. The contradictory results offered by some studies come from not differentiating the role of TReg cells at the two phases of sepsis. Tatura et al. [71] have studied for the first time the specific role of these cells during both, the early hyper-inflammatory and the immunosuppressive phase in a mice model of sepsis. The transcription factor forkhead box P3 (FOXP3) has been considered as an essential key gene involved in the development and function of TRegs. Intriguingly, TReg cells comprise natural TReg cells, in which expression of Foxp3 is constitutive, and induced TReg cells derived from native T cells and characterized by their plasticity and regulated Foxp3 expression. FOXP3 gene expression is also controlled by epigenetic mechanisms [72]. Specifically, it depends on DNA methylation levels and also on the levels of euchromatin marks at its promoter [73], in the so called TReg demethylated region (Foxp3-TSDR) [72]. Tatura et al. [71] have demonstrated that Foxp3-TSDR is demethylated in both natural and induced TReg cells during sepsis, a process that determinates the stability of these cells. They found that depletion of Foxp3 TReg cells leads to a more severe phenotype of sepsis, high levels of IL-6 and high mortality, suggesting that TReg cells can attenuate the hyper-inflammatory response in the first phase of sepsis, and unmethylated Foxp3 in TReg cells limits the hyper-inflammatory response and is related with a better outcome in mice [71]. However, during the immunosuppression phase and secondary infection with *Pseudomonas aeruginosa* pneumonia in a mouse model, Tatura et al. [71] did not observe changes in severity of sepsis, cytokine levels and mortality rates in mice with depleted Foxp3 TReg cell, suggesting that TReg cells may not have any influence in this phase of sepsis. On the other hand, Foxp3 acts as both activator and repressor of Foxp3-dependent genes transcription, and its binding to these genes depends on the levels of H3K27me3 and H3K4me3 [74]. Even more, TReg cells can be defined as a T cell subpopulation possessing a specific hypomethylated DNA pattern [75]. These data suggest that TReg transcriptional programs depend on the epigenetic programs of Foxp3-dependent genes, and loss of Foxp3 in TReg cells reestablishes their ability to produce immune effector cytokines (i.e., IFNγ, IL-4 and IL17) [76].

The epigenetic status of TReg cells make them good candidates for the monitoring of their function during pathological immune processes [77], mainly during the hyper-inflammatory phase.

B-lymphocytes

B cells or B-lymphocytes produce cytokines and have a relevant immunoregulatory role presenting antigens to T lymphocytes, and differentiating into antibody producing cells [78]. B cell exhaustion is also a hallmark of sepsis and it compromises the ability of B cells for production of antibodies and the efficient eradication of pathogens. Furthermore, a role for B cells has been suggested in post-septic immunosuppression [79]. Several epigenetic mechanisms are involved in the maturation, development and regulation of B cells, as well as the maturation of the antibody response. During B cell development in the bone marrow, DNA methylation and changes in post-translational modifications in histones participate during the V(D)J recombination process for the rearrangement of immunoglobulin heavy (IgH) and light (IgL) chains [80]. Furthermore, microARNs seem to be essential for B cell development and function [81]. miR-17-92 cluster, miR-124

and miR-181a participate in B cell proliferation and apoptosis by targeting Bim and Pten transcripts [82-84]. miR-34a blocks the B cell development whereas its depletion increases the number of mature B cells [85]. Finally, epigenetic dysregulation in B cells, including the aberrant expression of non-coding RNAs and alterations of histone modifications and DNA methylation, may result in an aberrant antibody release against foreign antigens [86].

As described above, epigenetic imprinting occurs on mature immune cells, therefore conditioning the pro-inflammatory and anti-inflammatory response in septic patients. Epigenetic imprinting might also occur in progenitor cells in the bone marrow and in other immune tissues like spleen and thymus during sepsis, which may contribute to explain why the immune system is not completely recovered by the generation of new immune cells from the bone marrow. Taking this idea further, epigenetic reprogramming may be retained in the progenitor cells of patients who survive sepsis, allowing them to perpetuate the epigenetic marks into differentiated cells, compromising the immune response, as proposed by Carson et al. [11]. Although scarce information exists at this moment about the epigenetic reprogramming in progenitor cells, it is evident that the analysis of undifferentiated cells and immune tissues in post-septic survivors could clarify the imprinting suffered during sepsis. Due to the existence of this epigenetic imprinting, epigenetic programs (i.e., DNA methylation, histone PTMs and microRNA signatures) can be studied in immune cells in order to obtain diagnostic and prognostic biomarkers, and to develop therapies aimed to improve the late deaths of sepsis and the long-term survival of affected patients.

The histone proteins as mediators of immune cells death

Histones and other nuclear components are released to the circulation as a result of extensive cell death, and if not rapidly cleared, their presence has deleterious effects on the endothelium of blood vessels which can be further extended to other tissues. As a result, several pathologies in which an inflammatory response together with tissue damage concur are characterized by the presence of circulating histones in blood plasma, although the concentration of histones and other nuclear material are differentially involved in the progression of the specific pathologies. In the latest years, extracellular histones have become an important part of the damage-associated molecular pattern (DAMP) response in septic processes as well as in sterile inflammation [87-89]. During the first hours of sepsis most neutrophils undergo apoptosis [40]. The presence of histones in pathologies that concur with a prolonged inflammatory response - as is the case of sepsis - is not only due to tissue damage, but also to a second source: activated neutrophils generate NETs, structures made of cellular components which include specifically modified histones. Generation of circulating histones from NETs or from necrotic neutrophils implies the release of a high concentration of histones to the bloodstream. Both processes, NETosis of neutrophils and necrosis of neutrophils and

other immune cells, contribute to the pathogenesis of sepsis. In parallel, the coagulation system, which is closely tied to these neutrophil cell death mechanisms, is often over-activated [90]. The major components of NETs included in the web structure consist of chromatin and histones, which are recognized initiators of disseminated intravascular coagulation (DIC) [91], are also related with poor prognosis in sepsis.

Early works established the capacity of histone preparations to induce cellular death in bacteria and mammalian cell models [92]. This antimicrobial role has, however, the secondary effect of compromising the viability of the nearby endothelial cells in the blood vessels, which in turn, after dying, release their own histones to the circulation. Thus, the generation of a feedback process that increases circulating histone levels in plasma exacerbates the deleterious effect of a sustained inflammatory response, and underlies many of the systemic tissue damage events during progression of sepsis. Histones are normally cleared from circulation by the liver, but an extensive production as that observed in sepsis or severe trauma overcomes the body's capacity to remove histones. Cumulative evidence points to the production of histones as a major determinant of tissue damage; their presence in the blood plasma of autoimmune disorders like rheumatoid arthritis or systemic lupus erythematosus (SLE) has been deeply studied [93], but in the latest years their presence in different types of cancer and other pathologies has also been reported. For instance, recent works have shown the relevance of histones and nucleosomes extensively produced in pancreatitis, as a result of extensive acinar cell death [94, 95]. Histones have also been defined as inflammatory mediators underlying the inflammatory response produced after radiofrequency ablation in hepatic carcinoma [95]. Kawai et al. [96] presented recently a thorough study in which administration of histones to mice permitted a precise follow-up of the subsequent multiple organ failure.

Regarding sepsis, circulating histones are strongly related with specific organ failure and mortality [95, 97]. Hence, the specific determination of pathological histones levels, and the development of diagnostic tools that permit a fine monitoring of their clearance or accumulation in blood plasma from patients are becoming critical goals for the treatment of sepsis.

Molecular mechanisms underlying histone cytotoxicity

Although evidences for histones released to the circulation, and their deleterious effect on endothelium and other tissues have been thoroughly described, the molecular mechanisms underlying histones toxicity remain largely unresolved. It is known that histones can bind the plasma membrane of cells, possibly due to the highly positive surface of their globular structure. Several works have shown that they can non-specifically bind membrane phospholipids [98, 99] and promote the formation of pores that alter calcium influx, producing membrane depolarization [100]. However, the molecular chain of events that produce cell death from the histone-membrane interaction is not fully understood. Histones produce not only necrotic-like [55] but also apoptotic-like cellular death [101]. In this latter sense, involvement of caspase-3

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seems to point out a specific death pathway induced by histones, but several works seem to suggest that TLRs are critical for the specificity and progress of the histone-mediated cell damage. In this regard, TLR2 and TLR4 have been functionally related to cell death observed in sepsis and fatal liver injury models [102, 103]. It is also interesting that histones, at concentrations lower than that triggering cell death, affect progenitor endothelial cells reducing their capacity to differentiate and regenerate into tubules, arresting their cell cycle. The involvement of the TLR2 and TLR4 receptor pathways in this process could be mediated by p38 MAPK, as proposed in [104].

Toxicity for all histone types has been described, but some data are controversial in this regard: Abrams and collaborators obtained similar cytotoxicity results for both interlinker H1 and core H2A, H2B, H3 and H4 histones [55], whilst other authors are coincident in finding higher cytotoxicity for H3, H4 and in some cases H2B, rather than H2A [89, 104]. Histones mediate the apoptosis of cells in the lymphoid compartment observed in the thymus [45], spleen [46] and blood [47], compromising the adaptive immunity. Particularly, H4 has been proposed as the main histone driving lymphocyte apoptosis. Therefore, neutralization of this histone by means of immunotherapy has been proposed as a potential therapy in clinical interventions against sepsis [81]. Other studies have identified histone H3 and nucleosomes in plasma from septic patients [16, 61, 83]. Structural properties of histones, such as the predominance of histone-fold structural regions (which are absent in H1 and significantly different in H2A) could explain the functional diversity on the effects of histones on cellular membranes. A capital question regarding histones' cytotoxicity is whether histone posttranslational modifications (PTMs) could affect the deleterious effect of their release to blood plasma or other biological fluids. Several works point to specific roles for certain histone variants and modifications in relation to their participation in human disease, but there are not strong evidences that could provide an insight into which PTMs on histones are more related to cellular damage. It has been shown, for instance, that glyoxidation products of histone H2A are involved in the progression of cancer [105]; citrullination of histones is required for the correct formation of NETs [106], and the presence of antibodies specific for citrullinated histone types have been found in several autoimmune disorders [48]. Citrullination of histone H3 during NETosis is catalyzed by PAD-4 [107]. Furthermore, citrullination of histones, in particular histone H3, was revealed as a convergence point for diverse inflammatory signals that trigger the neutrophil response to infections being a potential serum biomarker for the early diagnosis of septic shock [108].

Extracellular citrullinated H3 has also been found in pancreatic cancer, and recent evidence suggests the involvement of autophagy pathways in the correct generation of NETs [109]. Other autoimmune disorders such as Felty's syndrome have provided interesting clues about the specific antigenic determinants related to chromatin, NETs and particular deiminated histone types [50]. It has recently been described how NETs from SLE patients contain many methylated and acetylated residues in their histones [110]. In this context, when neutrophils undergo NETosis, specific residues of histones associated to apoptosis

were more susceptible to acetylation (i.e., H4K8, H4K12, H4K16 and H2BK12) and methylation (H3K27). The hyperacetylation and hypermethylation of these specific residues on histones from NETs in SLE is suggested to increase the immunostimulatory potential of NETs [110]. It would be interesting to demonstrate if a similar process occurs during sepsis. Nonetheless, whether modification of histones is merely related to NET formation or a consequence of the loss of homeostasis that leads to necrotic cell death, as well as the differences in cytotoxicity for specific histone modifications have never been described.

In summary, circulating histones are becoming critical molecular biomarkers in pathologies that concur with inflammatory exacerbated response and diverse tissue damage, and bear promising value as progression and prognosis reporters in the multiorgan failure of prolonged sepsis. Dissecting the specific toxicity of each histone type and the limitant concentration values that might trigger extensive tissular damage lay at the center of research focused on biomarker development for a precise sepsis prognosis.

Therapies based on epigenetic drugs

Lower levels of circulating cytokines have been detected in patients suffering from sepsis, in contrast with high levels observed in animal models of sepsis. This partially may explain why some of the treatments that function in animal models have failed after application in humans [111]. Despite considerable investigation, no clear therapies to modulate the inflammatory response in sepsis have emerged. More than 30 clinical trials consisting on the use of anti-inflammatory agents have failed. Therefore, these results do not support the hypothesis the inflammation is the main component of the fatal onset of sepsis, although inflammation clearly has a key role in the first phase of sepsis. In this sense, sepsis compromises the function of almost all types of immune cells (neutrophils, myeloid-derived suppressor cells, CD4+ and CD8+ T Cells, as well as B cells). Moreover, immunosuppression and the mechanisms involved in the dysregulation of anti-and pro-inflammatory genes may have a relevant role in the fatal onset of sepsis.

In sepsis, extranuclear circulating histones which, as described above, are released abundantly during NETosis, can be detected in blood [112]. Since circulating histones are also harmful for the host cells [102], histones should be targets of new therapeutic efforts. Several strategies are being developed to counteract the production of plasma histones, such as treatment with low-molecular-weight heparins or other proteins that bind and remove histones from the circulation [53, 88, 113] or by means of specific antibodies [7]. For example, neutralization of H4 using specific antibodies has been proposed as a potential therapeutic approach in clinical interventions to avoid lymphocyte apoptosis [81]. However, these strategies may contribute to by-pass pathological complications or poor prognostic in sepsis, instead of preventing late immunosuppression in septic patients. Despite these advances, new territories related to histone post-translational modifications and epigenetics should be explored in order to battle against long-term immunosuppression and avoid deaths over time in post-septic patients. Synthetic compounds

have appeared to interfere with the recognition of acetylated histones with bromodomain and extra terminal domain (BET) family proteins (BRD2, BRD3, and BRD4). These proteins regulate inflammatory gene expression throughout the assembly of histone acetylation [114, 115]. Interestingly, in vitro results have demonstrated the treatment of bone marrow-derived macrophagues with BET inhibitors, specifically the compound I-BET GSK525768A, affect the levels of H3ac, H4K5ac, H4K8ac, H4K12ac and total H4ac in the promoters of genes induced by LPS challenge. The promoters affected were at genes such as cytokines and chemokines (i.e. IL-6, IFN-b1, IL-1b, IL-12a) and the expression of transcription factors Rel, IRF4 and IRF8 participating in the initial wave of inflammatory gene expression was also affected by BET inhibitors [116]. In addition, these inhibitors seem to have a therapeutic effect in sepsis and endotoxic shock, not only regulating the expression of inflammatory gens but also TNF-inducible pro-inflammatory genes, chemokine genes, as well as vasoactive genes that contribute to sepsis complications [116]. However, the results obtained by BET inhibitors suggest that their use could only have beneficial effects on the phase of overwhelming inflammatory response, during the first days of sepsis.

A strategy to improve clinical outcome during the phase of late immunosuppression and to avoid the apparition of opportunistic infections could consist on a therapy based on IFN-γ [117]. IFN-γ is an essential cytokine for immunity against intracellular pathogens which improves monocytic function in septic patients deficient in HLA-DR expression and with compromised ability to produce LPS-induced TNF-α *in vitro* [117-119]. Since epigenetic mechanisms underlie CD8+ T lymphocyte differentiation, targeted epigenetic therapies could contribute to IFN-γ production by CD8 T+ lymphocytes [120, 121], which in turn may activate macrophages. Chang and Aune [122] described the high dynamisms of the epigenetic control at the IFNG locus during T cell differentiation. During TH1 cell differentiation, methylation of H3K9 is maintained but decreases in TH2 cells, contributing to the recruitment of Stat6 and Gata3 transcription factors and the methyltransferase EZH2, leading to an increase of H3K27me3 levels and facilitating TH2 differentiation [121, 122].

Recent findings, have shown epigenetic therapy might control immune response in three cancer types (breast, colorectal and ovarian cancers). In this regard, Li et al. [123] have demonstrated the immune stimulatory potential of the DNA methyltransferase inhibitor 5-azacytidine (5-AZA) by detecting an enrichment of immunomodulatory pathways, including in interferon (α, β and γ) signaling, antigen presentation and processing, viral RNA destruction and cytokine/chemokine expression. The genes controlling these pathways and affected by 5-AZA were termed AZA Immune Genes (AIMs). Furthermore, Li et al. [123] also demonstrated the use of the histone deacetylase inhibitor Trichostatin A (TSA) also upregulates a significant number of AIMs, in agreement with previous results in which HDAC inhibitors produced important effects on the host immune system responses [124].

A therapeutic approach to reestablish IFN-γ production by CD8 T+ cells is the administration of 5-AZA, which has been shown to increase IFN-γ production in cell culture supernatants of CD8 T+ lymphocytes [125], as well as an increment in the number of native CD8 T+ cells able to produce this cytokine when compared to control [126]. Another possibility may be the use of inhibitors of the specific histone methyltransferase G9a, which may contribute to sustain the levels of H3K9me3 at the IFNG locus and to maintain TH1 specific transcription factors. This hypothesis might be supported by the observations of El Gazzar et al. [127]: the authors proposed that G9a depletion contributes to the activation of TNF-α promoter and transcription, which is also in a narrow cross-talk with IFN-γ in TH1 cells [128]. This strategy may improve immunological responses during the immunosuppression phase after a septic episode.

Conclusion

The immune landscape of sepsis is complex, although it seems to be orchestrated by molecular pathways and epigenetic forces that control both "pro-inflammatory" and "anti-inflammatory" phases, thus mediating the immunosuppression of patients after a sepsis episode. Both phases occur steeply throughout the progression of sepsis, with the early phase of sepsis evolving with an important participation of pro-inflammatory pathways, and the latter phase of sepsis characterized by immunosuppression, which conduces to development of secondary infections or virus reactivation, increasing the probability of late-deaths. It is still not clarified the impact of immunosuppression over the long-term prognosis but everything points to a key role.

In these processes, epigenetic mechanisms are the conductors of specific chromatin signatures and transcriptional programs in immune cells, therefore conditioning the pro-inflammatory and anti-inflammatory responses in septic patients. Epigenetic imprinting may not only occur in differentiated innate and adaptive immune cells, which may explain late immunosuppressive process, but also in progenitor cells in the bone marrow and in other immune tissues like spleen and thymus during sepsis, which could contribute to long-term immunosuppression and long-term deaths of survivors from sepsis.

Many questions on the cytotoxic effect of histones and the particular role of histone types and PTMs that condition immunosuppression make it extremely difficult to develop precise and effective biomarker and therapeutic strategies. The comprehension of epigenetic mechanisms and specifically the role of diverse histone types and PTMs will help to clarify the epigenetic control of genes participating in the pro-inflammatory and anti-inflammatory phases orchestrating immunosuppression, and hence will allow developing precise and effective diagnostic and prognostic biomarkers. There exists a perception that the recovery or preservation of the host immune function would contribute to improve survival in sepsis, especially in late deaths produced by immunosuppression. Therefore, therapeutic strategies that enhance the immune system may clearly contribute to the management of septic patients and probably improve the physiological state of patients affected by long-term immunosuppression. In this regard, epigenetic drugs and their regulative effect on the expression of key immune genes should play a pivotal role for future investigations and clinical trials.

Acknowledgement

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