Monitoring Immune Dysfunction in Septic Patients: Toward Tailored Immunotherapy

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Introduction

Septic syndromes represent a major although largely under-recognized healthcare problem worldwide accounting for thousands of deaths every year [1–3]. Mortality remains high ranging from 20 % for sepsis to over 50 % for septic shock despite almost 20 years of anti-inflammatory clinical trials [1–3]. The inability of these therapies to mitigate the devastating effects of this condition indicates that the initial hypotheses for sepsis pathophysiology may have been misconstrued or inadequately addressed. Two major explanations have been proposed: 1) Septic patients have mainly been treated as a group despite the extreme heterogeneity characterizing this population [1]; 2) The postulate that death after sepsis is solely due to an overwhelming pro-inflammatory immune response may actually be inaccurate [1, 3]. Indeed, several lines of evidence have now established that death from septic shock is probably due to the effect of distinct mechanisms over time [1–3]. Early in the course of the disease, a massive release of inflammatory mediators (normally designed to trigger an immune response against pathogens) is occurring that may be responsible for organ dysfunction and hypoperfusion [1, 3]. Concomitantly, the body develops compensatory mechanisms to prevent overwhelming inflammation and dampen an overzealous anti-infectious response [1–3]. These negative feedback mechanisms, although having protective effects during the first initial hours, may paradoxically become deleterious as they persist over time leading to immune paralysis (**Fig. 1**) [1, 3]. Indeed, considerable clinical and experimental evidence indicates that patients rapidly present with numerous compromised immune functions [1, 3]. As our capacity to treat patients during the very first hours of shock has improved (early and aggressive initial supportive therapy) [1], many patients now survive this critical step but eventually die later in a state of immunosuppression that is illustrated by difficulty fighting the primary bacterial infection and decreased resistance to secondary nosocomial infections [1, 3]. Consequently, immunostimulatory therapies are now considered as an innovative strategy for the treatment of sepsis [1, 3]. However, the first critical step is to be able to identify patients who would actually benefit from these therapies [2, 3]. Indeed, in the absence of specific clinical signs of immune status, it is critical to determine the best biological tools to stratify patients according to their immune status (a missing step in most previous clinical trials) [1–3]. This would define the right action (i.e., stimulating innate immunity and/or adaptive immunity, blocking apoptosis, restoring other altered functions) at the right time (early or delayed treatment) in the right patient (individualized/tailored therapy).

Although the mechanistic and molecular bases for sepsis-induced immunosuppression are not exhaustively established, several features of the condition have been
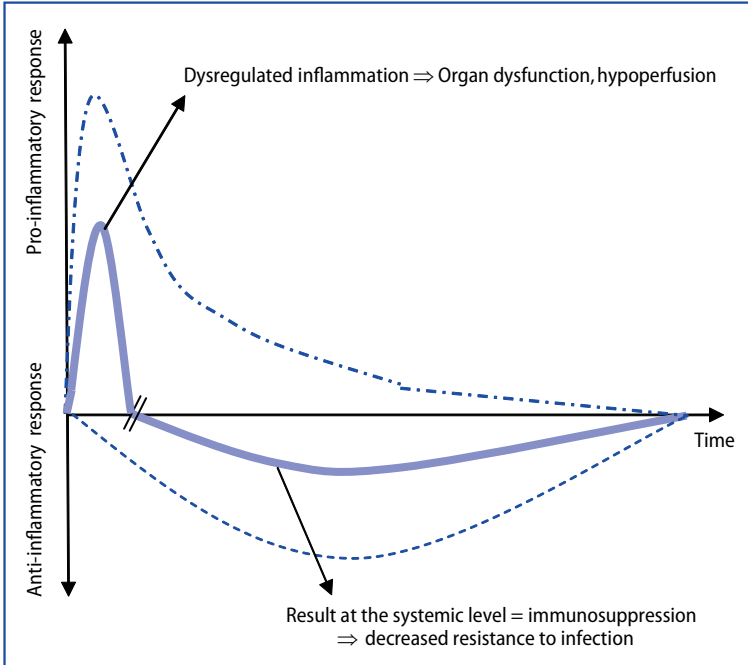
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Fig. 1. Simplified description of systemic pro- and anti-inflammatory immune responses over time after septic shock. Dashed lines: pro- or anti-inflammatory responses; bold line: result at the systemic level. The shift from a pro-inflammatory to an anti-inflammatory immune response predominant at the systemic level likely occurs within 24 hours after the diagnosis of shock.

already described including enhanced leukocyte apoptosis, lymphocyte anergy, and deactivated monocyte functions [1, 3]. This review will focus on the immune dysfunctions described so far in septic patients regarding monocytes and T lymphocytes (as examples of innate and adaptive immune cells) and their potential use as biomarkers on a routine standardized basis for prediction of adverse outcome or occurrence of secondary nosocomial infections and for guidance of putative immunotherapy.

Monocyte Dysfunction

Monocytes from septic patients are mainly characterized by a decreased capacity to mount a pro-inflammatory reaction upon secondary bacterial challenge and by impairment in antigen presentation likely due to the lowered expression of major histocompatibility class II molecules (MHC class II).

Functional Testing

Since it directly measures *ex vivo* the capacity of a cell population to respond to an immune challenge, functional testing represents the gold standard method to establish immune alterations. Several groups have investigated the capacity of septic

patients' monocytes to release pro-inflammatory cytokines in response to lipopoly-saccharide (LPS), other Toll-like receptor (TLR) agonists, or whole bacteria *in vitro* (see recent review in [4]). These tests represent reliable methods to assess the phe-nomenon of endotoxin tolerance defined as a reduced responsiveness to a secondary LPS challenge following a first inflammatory response [4]. Monocytes from patients usually present with a diminished capacity to release tumor necrosis factor (TNF)-α, interleukin (IL)-1α, IL-6, IL-12, whereas the release of anti-inflammatory mediators (IL-1 receptor antagonist [IL-1ra], IL-10) is not affected or is even slightly increased [4]. This observation shows that LPS can still activate monocytes but that the intra-cellular signaling pathways have been turned to favor production of anti-inflamma-tory molecules, therefore supporting the concept of leukocyte reprogramming [4]. However, although this test is considered as a good method to assess monocyte hyporesponsiveness after sepsis, it is not suitable for routine analysis/diagnosis.

Cell Surface Marker Expression

In terms of molecules expressed on monocytes, which are readily measured by standardized flow cytometry protocols, numerous studies have been performed regarding the measurement of HLA-DR (human leukocyte antigen-DR). In septic patients, decreased cell-surface expression of HLA-DR has regularly been observed on circulating monocytes [3]. As opposed to assessment of circulating mediators, the major advantage of measuring a cell surface marker such as mHLA-DR is that its level of expression is the result of the sum of the effects of multiple mediators that may all be regulated during septic shock. For example, mHLA-DR expression has been shown to be positively and negatively regulated by cytokines such as interferon (IFN)-γ and IL-10 as well as by corticoids and catecholamines [3]. There is now a general consensus that a diminished mHLA-DR expression is a reliable marker for the development of immunosuppression in critically ill patients [3]. Indeed, the decrease in mHLA-DR expression has been assessed as a predictor for septic complications after trauma, surgery or pancreatitis. In these studies, low lev-els of mHLA-DR (< 40 % of positive monocytes, normal > 90 %) were observed in patients who subsequently developed nosocomial infections [3]. In contrast, in injured patients with uneventful recovery, mHLA-DR rapidly returned to normal values (in general in less than 1 week). Similar results in burn patients and after septic shock also indicate that a low mHLA-DR expression is associated with sec-ondary septic/nosocomial events [3, 5]. Finally, decreased mHLA-DR has been shown to be predictive of adverse outcome in different groups of critically ill patients. This has recently been observed in burn patients [5] and after severe sep-sis and septic shock [3].

Mechanisms Responsible for Monocyte Dysfunction

Among all the cytokines released by monocytes and increased after sepsis, IL-10 is the sole cytokine to consistently correlate with mHLA-DR values [6]. IL-10 produc-tion is increased after sepsis and has been shown to predict mortality [1, 3]. Given its properties in suppressing the synthesis of numerous pro-inflammatory cytokines [7], this continued release may contribute to the immune dysfunctions observed after septic shock and thus may augment susceptibility to secondary microbial inva-sion [7]. Gogos et al. showed that IL-10 and IL-10/TNF-α ratios, among a panel of various cytokines (IL-1, IL-6, soluble TNF receptor [sTNF-R] I and II), were the

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most powerful predictors of mortality in patients with severe sepsis both at admission and 48 hours later [8]. We extended these results in a group of 38 patients by illustrating that IL-10 levels remained higher in non-survivors until 15 days after the onset of septic shock [6]. In particular, high IL-10 concentrations at the beginning of shock were negatively correlated with the nadir of mHLA-DR measurements during the 2 weeks of monitoring [6]. Consequently, this initial value of IL-10 may reflect the severity of the forthcoming immunoparalysis.

Increased monocyte apoptosis has also been described after sepsis [9, 10]. Adrie et al. observed that septic patients exhibited an increased percentage of monocytes with depolarized mitochondria (as a marker of apoptosis) when compared with healthy individuals [11]. Furthermore, among septic patients, this percentage was significantly higher in non-survivors than in survivors. However, one limitation in assessing the incidence of monocyte/macrophage apoptosis is that some of these changes may represent an increased role in clearance of apoptotic cells, which may make these cells look overtly more apoptotic as a result of handling a greater amount of apoptotic material [10]. With that said, the down modulation of CD14 expression on monocytes after septic shock (a cell surface marker decreased during monocyte apoptosis) tends to confirm this increased apoptotic process especially because its downregulation was more pronounced in patients who were not going to survive [12].

Restoration of Monocyte Functions

Based on the above, several innovative immunotherapies may be proposed (**Table 1**).

AS101, with the capacity to inhibit IL-10, has been demonstrated to increase survival in septic mice [13]. Although it can be argued that blocking a single mediator in a context where many inhibitory pathways are involved/activated, may remain inefficient [2], this molecule has been shown to act through different mechanisms (inhibition of IL-10, activation of macrophage functions, inhibitor of IL-1beta converting enzyme) and, therefore, remains a valuable potential therapeutic strategy [3].

Table 1. Monocyte dysfunction in the septic patient: Potential biomarkers and therapies

	Biomarker	Technique	Targeted therapy
Functional testing	↓ *ex vivo* cytokine production after TLR agonist stimuli	ELISA or CBA	GM-CSF G-CSF IFN-γ
Cytokines	↑ plasma IL-10	ELISA or CBA	AS101
Cell surface marker expression	↓ mHLA-DR ↓ CD14, CD86, GM-CSF, CX3CR1 ...	FCM	GM-CSF G-CSF IFN-γ
Apoptosis	Depolarized mitochondria ↓ CD14	FCM	GM-CSF G-CSF IFN-γ

TLR: Toll-like receptor; ELISA: enzyme-linked immunosorbent assay; CBA: cytometric bead array; HLA-DR: human leukocyte antigen-DR; FCM: flow cytometry; IFN: interferon; G-CSF: granulocyte colony-stimulating factor; GM-CSF: granulocyte macrophage colony-stimulating factor; IL: interleukin

Several molecules have already been used (IFN-γ, granulocyte-colony stimulating factor [G-CSF], granulocyte/macrophage-colony stimulating factor [GM-CSF]) to stimulate monocyte functions with interesting preliminary results *ex vivo* (increased mHLA-DR expression, restoration of cytokine production) [1, 3]. Several prospective randomized multicenter clinical trials using IFN-γ have been conducted in trauma patients [1, 3]. However, despite interesting results regarding secondary endpoints in some subgroups of patients (decreased severity of nosocomial infections, decreased mortality in infected patients), the results were inconclusive in terms of overall mortality or infection rates [1, 14–15]. Presneill et al. [16] published preliminary data regarding the use of GM-CSF in 10 patients with sepsis-induced respiratory failure. These authors observed a modest improvement in gas exchange, resolution of acute respiratory distress syndrome (ARDS) and alveolar leukocyte phagocytic functions, but no enhanced survival rate. In a prospective, randomized, placebo-controlled trial, Rosenbloom and colleagues investigated whether GM-CSF treatment could improve leukocyte function and mortality in 40 septic patients [17]. These investigators observed a higher leukocyte count, increased mHLA-DR, and better resolution of infections in the treated group but again no difference in mortality. Nevertheless, it should be noted that these trials were designed without patient stratification and drug efficacy should be assessed only in patients with prior established impairment in monocyte function.

T Lymphocyte Dysfunction

Due to their ability to interact not only with cells of the innate immune system but also with other cells of the adaptive response, T lymphocytes play a central role in the anti-infectious immune response both as effectors and regulators of this response [1]. This role is illustrated by the description of increased mortality, decreased bacterial clearance, and an altered pro-inflammatory immune response after polymicrobial septic challenge in mice lacking both T and B cells [1, 18]. A growing body of evidence has now confirmed that the lymphocyte-mediated immune response may be dysfunctional after severe sepsis and may play a major role in the development of a state of immunosuppression in such patients [1, 18].

Functional Testing

Lymphocyte anergy is illustrated by the observation of the loss of the delayed-type hypersensitivity reaction to recall skin tests antigens in patients [1, 18]. This loss of hypersensitivity has been well described and is known to be associated with mortality and with the development of secondary infections [1, 18–19]. Indeed, a marked decrease in lymphocyte proliferation in response to antigens (tuberculin, tetanus toxin) or non-specific (phytohemagglutinin, concanavalin A, anti-CD3, anti-CD28 antibodies) stimulation has been described in patients after severe injuries (sepsis, major surgery, severe burn or trauma) [1, 19]. Most importantly, it has been observed that the severity of this state of anergy correlates with poor outcome [1, 18], increased occurrence of infectious complications, and subsequent multiorgan failure in patients [18, 19]. The measurement of cell proliferation *in vitro*, usually performed with peripheral blood mononuclear cells (i.e., lymphocytes and monocytes), investigates the capacity of lymphocytes to proliferate and of monocytes to present antigens (when performed with recall antigens) in a single test. Proliferation

is usually assessed by ^{3}H-thymidine uptake or more recently by the use of fluorescent probes (like CFSE). However, as proliferation tests require long incubation times (2–3 days for mitogens, 5–7 days for recall antigens), they are not suitable for clinical decision-making and are not performed on a routine basis.

Cell Surface Marker Expression

T lymphocytes have been characterized by the overexpression of inhibitory co-receptors during immunoparalysis. It has recently been demonstrated in trauma patients that anergic T cells presented with increased programmed death-1 (PD-1), CD47 and cytotoxic T-lymphocyte-associated antigen-4 (CTLA4) expression that would facilitate preferential triggering of negative signaling pathways during T-cell stimulation [20] and, therefore, lead to lymphocyte anergy. Moreover, this increase in co-repressor receptors (in particular CTLA4) appears to be associated with a decrease in the expression of co-activator receptors such as CD28 or CD3, which could also play a role in the development of immunoparalysis [18, 21]. However, in contrast with mHLA-DR, only fragmental data are available regarding the correlation between these markers of lymphocyte suppression and mortality/morbidity and the development of nosocomial infections in patients.

Finally, one likely major characteristic of T lymphocyte dysfunction after severe injury is the increase among patients' circulating lymphocytes of a cell-population with known regulatory properties [18]. CD4$^+$CD25$^+$ regulatory T lymphocytes (Treg) have recently been reported as a potent regulatory T cell lineage playing an essential role in the control of both adaptive and innate immune responses [18]. An increase in the percentage of Treg has been described in septic shock patients [22]. Importantly, this increase was observed immediately after the diagnosis of sepsis; however, it persisted only in non-surviving patients in association with an augmented CTLA4 expression. A similar increase in Treg percentage has been observed in trauma patients and in mice after polymicrobial septic challenge and stroke [18]. We recently observed a strong correlation between the increased Treg/effector ratio measured in whole blood after septic shock and the decreased proliferative response of patients' lymphocytes after mitogenic stimulation. This suggests not only that the measurement of the Treg percentage may represent a reliable marker for the diagnosis of lymphocyte dysfunction in patients but also that these cells may play a central role in the development of immunoparalysis after sepsis.

Mechanism Responsible for Lymphocyte Dysfunction

It is generally agreed that apoptotic cell death represents the major mechanism triggering sepsis-induced lymphocyte anergy/dysfunction [1–2, 10]. After sepsis and severe trauma, this has been shown to be associated with a marked decrease in the number of circulating lymphocytes that is correlated with the development of nosocomial infections in these patients [1, 10, 18].

Pioneering autopsy studies by Hotchkiss et al. disclosed a profound, progressive, apoptosis-induced loss of cells of the adaptive immune system in the spleen, blood and gut-associated lymphoid tissue of adults who had died of sepsis [23–25]. Although no loss of CD8$^+$ T cells or natural killer cells occurred, sepsis markedly decreased the levels of B and CD4$^+$ T cells [25]. This loss was especially important because it occurred during life-threatening infectious process, while clonal expansion of these cells might have been expected [25]. Accordingly, Le Tulzo et al.

observed a marked increase in apoptosis of circulating lymphocytes from septic shock patients compared with critically ill patients without sepsis and healthy volunteers [26]. This induced a profound and persistent lymphopenia associated with poor outcome [26]. Bilbault et al. observed a severe downregulation in the expression of the anti-apoptotic gene, Bcl-2, in circulating mononuclear cells from patients with severe sepsis [27]. This was associated with a reduced T-cell count and an increase in annexin-V labeling. Most importantly, immediately after the onset of severe sepsis this decrease was higher in non-survivors than in survivors. A second study by this group confirmed these results by measuring Bax/Bcl-xl and Bax/Bcl-2 ratios in septic shock patients [28]. However, one major limitation to the use of apoptosis measurements as markers for immunoparalysis may be the drawbacks inherent to this type of experiment, such as the need for rapid processing of the samples (especially regarding annexin-V staining) which is hardly compatible with their use in ICUs [29]. Furthermore, as methods used for studying apoptosis may often have a significant rate of false positive results (especially the deoxyuridine triphosphate nick-end labeling assay), it is recommended that apoptosis be established on the basis of two or more methods of detection, including DNA-hypoploidy, morphology, DNA laddering, annexin-V staining, active caspase-3 or mitochondrial permeability measurements [2, 10].

Restoration of Lymphocyte Functions

Augmenting T cell function and fighting lymphopenia may, therefore, represent a valuable therapeutic strategy after sepsis (**Table 2**). IL-7 is an essential cytokine for T lymphocyte development, survival, expansion and maturation in humans [30]. Phase I clinical trials in cancer and in patients infected with human immunodeficiency virus (HIV) have shown that T cell expansion can be achieved at doses that are well tolerated [27]. In line with these findings, ligands of co-activator receptors for effector T lymphocytes may also possess beneficial effects. As an illustrative example, recent results by Scumpia et al. have shown that anti-glucocorticoid-induced TNF receptor family related gene (GITR) agonistic antibodies were

Table 2. Lymphocyte dysfunction in the septic patient: Potential biomarkers and therapies

	Biomarker	Technique	Targeted therapy
Functional testing	↓ proliferation after antigenic or non-specific stimulation	^{3}H-thymidine uptake or CFSE probes	IL-7 IVIG
Cell surface marker expression	↑ inhibitory receptors: PD1, CTLA4, CD47… ↓ co-activator receptors: CD28, CD3 ↑ % Treg	FCM	Anti-GITR agonistic abs IVIG
Apoptosis	↓ T cell count ↑ Annexin V staining ↓ Bcl2 expression protein/gene Bax/Bcl-xl or Bax/Bcl2 ratios	FCM RT-PCR/FCM RT-PCR/FCM	IL-7 Caspase-inhibitors Ritonavir

CFSE: carboxyfluorescein succinimidyl ester; IVIG: intravenous immunoglobulin; FCM: flow cytometry; GITR: glucocorticoid-induced tumor necrosis factor receptor; RT-PCR: Real time polymerase chain reaction; IL: interleukin; Treg: CD4$^+$CD25$^+$ regulatory T lymphocytes

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able to restore lymphocyte proliferation, prevent CD3 down-modulation, decrease bacteremia, and increase survival in a mice model of sepsis [31]. Intravenous use of immunoglobin has also been proposed as an adjuvant treatment for sepsis. However, to date, its benefits remain unclear [1]. The authors of recent meta-analyses recommend conducting larger clinical trials with patient stratification [32].

Finally, strategies designed at blocking apoptosis, including caspase-inhibitors, overexpression of Bcl-2, and inhibition of Fas/FasL signaling, have demonstrated survival improvement in animal models of sepsis [10]. That said, so far no therapeutic strategy has been developed sufficiently to reach clinical use. An alternative may be provided by HIV protease inhibitors, the activity of which is partly mediated through anti-apoptotic effects. Administration of ritonavir improved survival in a murine model of sepsis, even when given after the onset of the disease [33]. As these protease inhibitors are well tolerated in patients, we may expect exciting possibilities in sepsis. A phase I trial is currently underway investigating the effects of these drugs to boost the immune system in healthy volunteers (www.clinicaltrials.gov NCT00346619). Of note, drugs aimed at blocking apoptosis may be used as adjunctive agents in association with molecules targeting monocytes or leukocytes.

Conclusion

Our understanding of the pathogenesis of sepsis has been oversimplified during the past few decades and, as a result, many clinical trials have addressed the pro-inflammatory side when there was no evidence that hyper-inflammation was dominant in patients. Several issues require further definition before we can gain a complete picture of events leading to immunosuppression (e.g., Are the major sepsis-induced
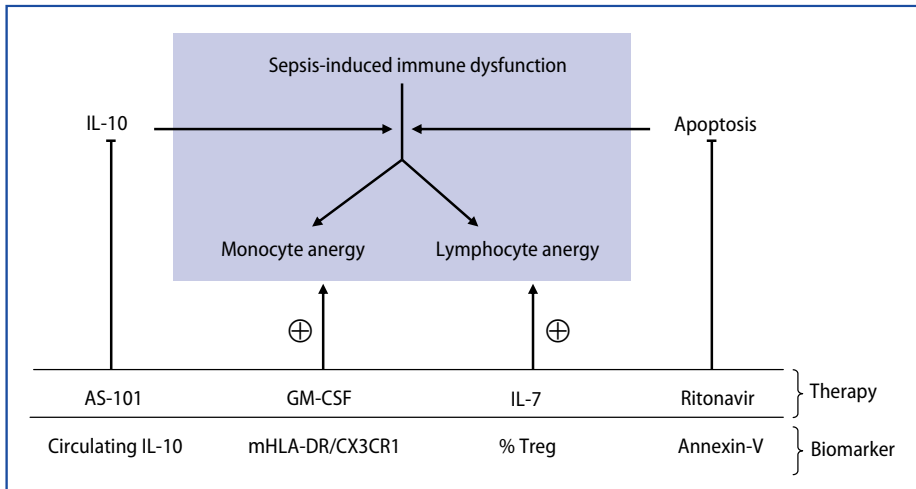

Fig. 2. Monitoring immune dysfunctions in septic patients: Toward tailored immunotherapy. Sepsis-induced immune dysfunction (lymphocyte and monocyte anergy) likely mediated by increased interleukin (IL)-10 and apoptosis might be treated by innovative and specific therapies administered based on measurements of selective biomarkers in patients before initiation of treatment. Treg: CD4$^+$CD25$^+$ regulatory T lymphocytes; GM-CSF: granulocyte macrophage colony-stimulating factor

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inhibitory mechanisms fully elucidated? How large a part is played by physician-induced immunosuppression, as sedatives, catecholamines, and insulin are all immunsuppressive agents? Is the cellular energetic status crucial in maintaining immune functions? How important is the neuroendocrine-mediated part of immunosuppression? How preponderant is immune failure among other organ failures? Could immune dysfunction be just another organ failure?). Nevertheless, we can reasonably state that patients with sepsis have features consistent with immunosuppression. Consequently, stimulating the patient's immune system may be a promising therapeutic strategy. Although we cannot predict which of these therapies will be efficacious, they surely deserve to be fully and minutely investigated when considering the high mortality that characterizes septic syndromes. However, so as not to repeat the mistakes of the past, an absolute prerequisite for future clinical trials is to systematically assess patients' immune functions prior to inclusion so as to be able to define individualized immunotherapy (**Fig. 2**).

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