LEADING ARTICLE

How to Identify Systemic Sepsis-Induced Immunoparalysis

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The body develops compensatory mechanisms to prevent systemic inflammation in response to stress and injury. As overwhelming inflammation is rapidly lethal, these mechanisms have a protective effect during the first few hours after injury. However, they become deleterious as nearly all immune functions are compromised. The term "immunoparalysis" describes the global incapacity of the body to mount any kind of immune response; the extent of immunoparalysis is thought to correlate with life-threatening secondary infections and mortality. The hypoimmune state might require proinflammatory therapies to enhance immune function, but establishing the presence of immunodepression is crucial when considering such an approach. This article discusses methods for diagnosing immunoparalysis, in particular measurements of circulating monocyte human leukocyte antigen type DR expression and plasma interleukin-10. *Advances in Sepsis* 2005;**4**(2)42–9.

Despite great improvements in critical care medicine over the past 20 years and numerous trials of anti-inflammatory drugs, sepsis, severe sepsis, and septic shock remain the leading cause of death in intensive care units [1,2]. Hypotheses of the pathophysiology of septic shock have evolved to take into account the disappointing results of many clinical trials, and it is now thought that death from septic shock may be due to distinct mechanisms that change as the condition progresses. At onset, septic shock is characterized by the overwhelming release of inflammatory mediators, which are responsible for organ dysfunction and hypoperfusion [3]. As sepsis persists, a shift towards an anti-inflammatory state is observed and patients develop features consistent with immunosuppression [4–9]. Importantly, it seems that the majority of shock-related deaths occur during this secondary hypoimmune state. Indeed, an ongoing study by Monneret et al. that has so far enrolled 120 patients with septic shock has observed that 70% of nonsurvivors are still alive 3 days after the onset of shock (personal communication, 2004). It has been proposed that patients who survive the initial hyperinflammatory response but subsequently die from sepsis are those who do not recover immune function. The mortality rate in these patients may be explained by their inability to clear the initial infection and a predisposition to nosocomial infection [4–9]. This may account for the failure of therapies aimed at blocking the inflammatory cascade [10]. The therapeutic window for initiating treatment with anti-inflammatory drugs is very narrow (likely to be <24 h), after which a treatment to increase immune function may be more beneficial (Fig. 1).

Two animal studies have demonstrated the complexity of treating sepsis. The first focused on tumor necrosis factor-α (TNF-α) therapy. Although this cytokine has been identified as one of the key inflammatory mediators of adverse effects during the first phase of septic shock, clinical studies of anti-TNF-α therapies have not demonstrated improved outcomes. However, therapy to increase TNF-α levels reversed sepsis-induced immunosuppression and improved survival in a murine model of cecal ligation and puncture (CLP) [11]. The second study found that blocking the anti-inflammatory cytokine interleukin-10 (IL-10) before the induction of sepsis in mice increased mortality, whereas blocking IL-10 12 h after CLP improved survival [12]. These studies illustrate the importance of timing, indicating that a different therapy may have effects at different times.

There appear to be two main causes of death during septic shock, which implies that two different complementary therapeutic options are needed. Prevention of early mortality due to severe organ dysfunction requires immediate and aggressive treatment as soon as the syndrome is identified, including early fluid therapy, vasopressors, drotrecogin alfa (activated), adrenal replacement therapy, and appropriate antimicrobial therapy [13–17]. Prevention of the delayed mortality due to a hypoimmune state may require

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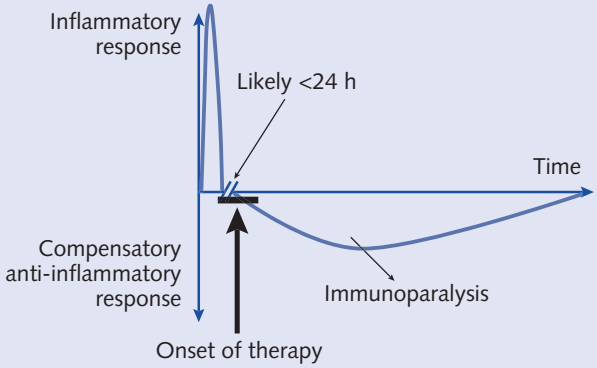
Figure 1. Simplified description of the pro- and anti-inflammatory responses after septic shock. At the onset of therapy most patients are already immunoparalyzed and anti-inflammatory drugs may be deleterious.



proinflammatory strategies to enhance immune functions. A key issue when considering such an approach is establishing the presence of immunodepression so as to identify patients who would benefit from immune stimulation. However, no clinical signs that characterize immune status have been identified to date.

Definition of systemic sepsis-induced immunoparalysis

In response to stress and injury (of which septic shock is a typical example), the body develops compensatory mechanisms to prevent systemic inflammation. This anti-inflammatory response is a homeostatic mechanism that occurs in all patients. It protects against lethal overwhelming inflammation during the first hours of the syndrome, but becomes deleterious as it persists because nearly all immune functions are compromised. As all cell types – neutrophils, monocytes/macrophages, and lymphocytes – are impaired, both innate and specific immunities are depressed. The terms "immunoparalysis" and "immunosuppression" have been proposed to describe the global incapacity of the body to mount any kind of immune response [18]. However, "systemic immunoparalysis" might be a better description of this phenomenon as pro- and anti-inflammatory responses are compartmentalized within the body. When local inflammation occurs, activating mechanisms that kill microbes, a parallel systemic response prevents the diffusion of inflammation [19,20].

It has long been established that patients with sepsis have features consistent with immunosuppression [4–9]. Briefly, the condition is characterized by:

- Impairment of neutrophil functions, including chemotaxis, phagocytosis, and bactericidal activity.
- Induction of apoptosis, inducing the loss of lymphocytes and dendritic cells.
- Hyporesponsiveness of lymphocytes, which become unable to proliferate or secrete cytokines in response to recall antigens.
- Reduced levels of Th1 cytokines (interferon-γ [IFN-γ]) and increased levels of Th2 cytokines (IL-4).
- Massive release of anti-inflammatory mediators, including cortisol, epinephrine, norepinephrine, IL-10, transforming growth factor-β (TGF-β), soluble TNF receptors, and IL-1 receptor antagonist.
- An increased proportion of regulatory T cells ($CD4^+$ and $CD25^+$), which produce IL-10 and TGF-β and induce lymphocyte anergy.
- Monocyte deactivation, defined by the inability to produce inflammatory cytokines, and diminished antigen presentation capacity, which is likely to be due to decreased expression of human leukocyte antigen type DR (HLA-DR) on the monocyte surface.

How to identify systemic sepsis-induced immunoparalysis

The ideal biological diagnostic tool

Sensitivity and specificity are the usual parameters taken into account when assessing a biological diagnostic tool. However, the lack of clinical signs associated with immunoparalysis makes the definition of true positives and false negatives virtually impossible. Moreover, there is currently no biological "gold standard" with which to compare a new diagnostic tool. Clinical studies are consequently required to correlate different markers with functional testing and to determine a link with outcome or septic nosocomial complications. Practical issues also need to be considered, such as analytical feasibility, reproducibility, time taken to complete the analysis, and, most importantly, the standardization of the assay to allow comparison of results between different centers and studies. Although the preliminary results of immunoparalysis monitoring studies are encouraging, these criteria are still unfulfilled.

Functional testing

Functional testing is the best means to establish the presence of immunoparalysis as it directly measures the capacity of a cell population to respond to an immune challenge *in vitro*. Cellular responses (such as cytokine production, cell proliferation, or cell-surface marker expression) to *in vitro* pharmacological or antigen challenges reflect *in vivo* functionality, and several assays have been developed during the last decade. However, these methods are not always suitable for routine monitoring as the need for purification and incubation makes them time consuming, and samples

cannot be stored. Another significant problem is the lack of standardization. Assays often include a cell purification step which may artificially activate cells and introduce variability between samples. Additionally, cell stimulation is often achieved using strong nonphysiological activators that may overwhelm the physiological capacity of the cell, producing erroneous results. Therefore, considering the high variability of human cellular responses in functional testing and the difficulties involved in standardizing tests between laboratories (the tests are poorly reproducible, with a variability of 50–100%), it is impractical to use these tools on a routine basis. However, although these functional tests are not suitable for everyday clinical decision-making, they remain essential to obtain insights into the pathophysiology of sepsis-induced immunodepression and to assess the validity of other, more easily measured and standardized, markers of immunoparalysis.

Measurement of circulating mediators

Although many studies have focused on the measurement of serum mediator levels, none has identified a universally accepted marker. One reason for this is that sepsis is caused by the interaction of extremely complex networks and amplification cascades: >200 rapidly changing mediators are involved. Furthermore, the host response is influenced by many factors, including the virulence of the invading microorganisms, bacterial load, the site of infection, coexisting conditions, and genetic predisposition. Elevated serum concentrations of Th1, Th2, and pro- and anti-inflammatory mediators have been correlated with an adverse outcome in various clinical contexts, but none is routinely used for patient monitoring. Although the inflammatory cytokines TNF-α, IL-6, and IL-8 have been extensively investigated over the past 2 decades, the results have not been conclusive and, in the context of immunosuppression, measurement of anti-inflammatory mediators may be more valuable. However, little work has been devoted to this area although many immunosuppressive mediators are known to be elevated during septic shock, including IL-10, TGF-β, IL-1-receptor antagonist, soluble TNF receptors, cortisol, catecholamines, prostaglandins, and various peptides.

The most consistent data are available for IL-10, which appears to be a predictor of fatal outcome. Gogos et al. showed that IL-10 levels and the IL-10/TNF-α ratio were the best indicators of mortality in a panel of cytokines [21], while van Dissel et al. reported that a high IL-10/TNF-α ratio was associated with poor outcome in a group of 450 febrile patients [22]. Moreover, in a separate study of critically ill septic patients, IL-10 levels were found to be higher in nonsurvivors at admission and provided a more accurate predictor of prognosis than IL-4 levels [23]. Monneret et al. recently demonstrated that IL-10 levels remained higher in nonsurvivors than survivors for 15 days after the onset of septic shock in a group of 38 patients [24]. Neither TNF-α nor TGF-β values differed between survivors and nonsurvivors. Interestingly, IL-10 was the only cytokine to correlate with monocyte HLA-DR (mHLA-DR) values. Specifically, high IL-10 concentrations early in sepsis were negatively correlated with nadir mHLA-DR measurements [24], suggesting that the initial IL-10 value predicts the severity of immunoparalysis. Lekkou et al. found similar results upon measuring cytokine and mHLA-DR levels in a group of patients with severe sepsis [25].

Given the ability of IL-10 to suppress the synthesis of numerous proinflammatory cytokines [26], its continued release may contribute to the immune dysfunction observed after septic shock and thus may increase susceptibility to continuous microbial invasion [26–28]. TGF-β is another potent immunosuppressive cytokine that is often implicated in immunoparalysis [4,5,8] and monocyte deactivation during endotoxin tolerance [29]. Monneret et al. found that, in contrast to IL-10 values, TGF-β levels were slightly above normal in both survivors and nonsurvivors [24]. This observation is in agreement with previous findings that although TGF-β levels are lower in septic patients, they do not significantly differ from control values [27,30]. Similarly, Lekkou et al. did not find any difference in TGF-β levels between survivors and nonsurvivors [25]. These results indicate that IL-10 is likely to be more important than TGF-β in the pathophysiology of sepsis-induced immunoparalysis. Similar findings have been observed in experimental studies. Anti-IL-10 antibodies were more effective than anti-TGF-β antibodies in reversing endotoxin tolerance in isolated monocytes [29]. In addition, Sfeir et al. found that anti-IL-10 antibodies inhibited the effects of plasma from septic patients on TNF-α release from normal monocytes, whereas anti-TGF-β antibodies did not [27]. Furthermore, the decreased expression of mHLA-DR due to endocytosis and intracellular sequestration of mature major histocompatibility complex class II molecules in septic patients has been shown to be mediated by IL-10 but not TGF-β [31]. Conversely, Le Tulzo et al. recently reported that increased circulating cortisol levels in patients with septic shock were correlated with low mHLA-DR expression, whereas IL-10 and catecholamines were not, suggesting that cortisol might be a useful additional marker [32]. Taken together, these results indicate that IL-10 is the most promising candidate as a marker of immunoparalysis. As it has been assessed only as a predictor of risk of death, it now needs to be investigated in larger clinical studies and correlated with functional tests.

The main advantage of using circulating mediators as markers of immune status is the reliability of the majority of the assays. They are standardized, reproducible (<10% variability), and in some cases are now automated, making them suitable for clinical decision-making. However, measurement of the concentration of only one or a few mediators remains questionable as it only provides a limited view of the patient's condition. In many cases, establishing the dominant response on the basis of serum measurements appears to be impossible as both pro- and anti-inflammatory responses are enhanced during septic shock.

Measurement of cell surface markers

As diagnostic tools, cell surface markers have the advantage that their level of expression is determined by multiple mediators. In addition, they can be measured in whole blood immediately after sampling with little manipulation of the cells prior to staining, thus minimizing the nonspecific activation that is associated with lengthy purification procedures. Several markers on different cell types have been assessed. A number of cell surface markers are known to vary during sepsis, but the data are currently too limited to draw firm conclusions. The largest body of literature is available for mHLA-DR expression, and it has been proposed that diminished mHLA-DR expression provides a reliable reflection of immunosuppression in critically ill patients, in terms of both its magnitude and persistence [7,33].

Expression of mHLA-DR in sepsis

mHLA-DR expression and functional parameters

Several studies have demonstrated an association between low levels of mHLA-DR expression and the impairment of different cellular functions (particularly antigen presentation), supporting the hypothesis that mHLA-DR is a global marker of immunoparalysis. Monocytes from septic patients with decreased levels of mHLA-DR have been shown to produce small amounts of TNF-α and IL-1 in response to bacterial challenges such as stimulation with lipopolysaccharide (LPS), staphylococcal enterotoxin B, and phorbol myristate acetate [30]. Another study showed that lymphocytes from septic patients with low mHLA-DR levels were unable to proliferate in response to tetanus toxoid, demonstrating a failure in antigen presentation [34]. Accordingly, Wolk et al. mimicked the decrease in mHLA-DR in an experimental model of endotoxin tolerance and showed the impairment of T cell proliferation and IFN-γ production in response to recall antigens such as tetanus toxoid, tuberculin, and candidin [35]. These data demonstrate that paralysis of monocytes not only diminishes their proinflammatory effect, but also reduces their capacity to induce antigen-specific T cell responses. In this situation, diminished mHLA-DR expression could be considered as a global reflection of impaired monocyte function. A recent investigation found that the perioperative administration of granulocyte colony-stimulating factor (G-CSF) increased expression of mHLA-DR in surgical patients [36]. This increase was accompanied by increased lymphocyte proliferation and Th1 cytokine production (IL-2 and IFN-γ) in response to phytohemagglutinin and an increased capacity for inflammatory cytokine release after LPS challenge in a whole blood model.

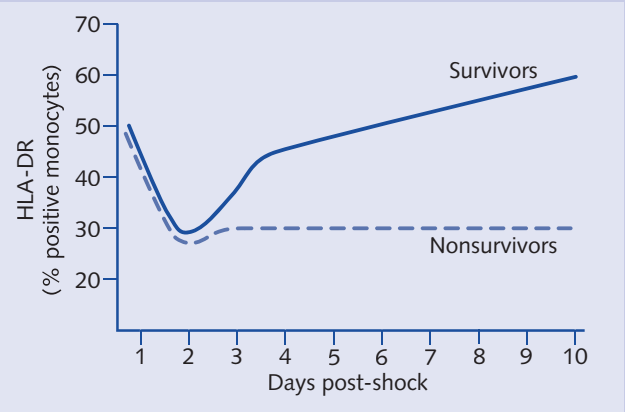
Clinical studies of mHLA-DR expression

To date, mHLA-DR levels have mainly been assessed as a predictor of septic complications after trauma, surgery, and pancreatitis [28,37–40]. Low levels of mHLA-DR were found in patients who subsequently developed nosocomial infections. In contrast, mHLA-DR levels normalized rapidly (generally within 1 week) in patients who recovered uneventfully. Diminished mHLA-DR expression has previously been identified as an independent predictor of septic complications in multiple logistical regression analysis after correction for clinical parameters [41].

The predictive value of mHLA-DR in patients with severe sepsis and septic shock is unclear. Although the reduction in mHLA-DR in sepsis is generally well accepted, some studies have not found it to be correlated with mortality [31,42,43], whereas others have [23,25,44–46]. However, these preliminary studies included small numbers of patients, and monitoring was generally limited to the first 48 h after the onset of sepsis. In contrast, Monneret et al. observed that the difference in mHLA-DR values between survivors and nonsurvivors became significant after 48 h [46]. The persistence of low (<40%) mHLA-DR expression for 5 days after septic shock was found to be associated with a fatal outcome. The nadir of mHLA-DR expression appears to occur around the second day after shock, but there does not appear to be any measurable difference in mHLA-DR values between survivors and nonsurvivors at this point. In survivors, mHLA-DR values subsequently increase, whereas they remain low in nonsurvivors (Fig. 2). Thus, the different sampling time points used in the various studies may explain why mHLA-DR values did not consistently predict mortality.

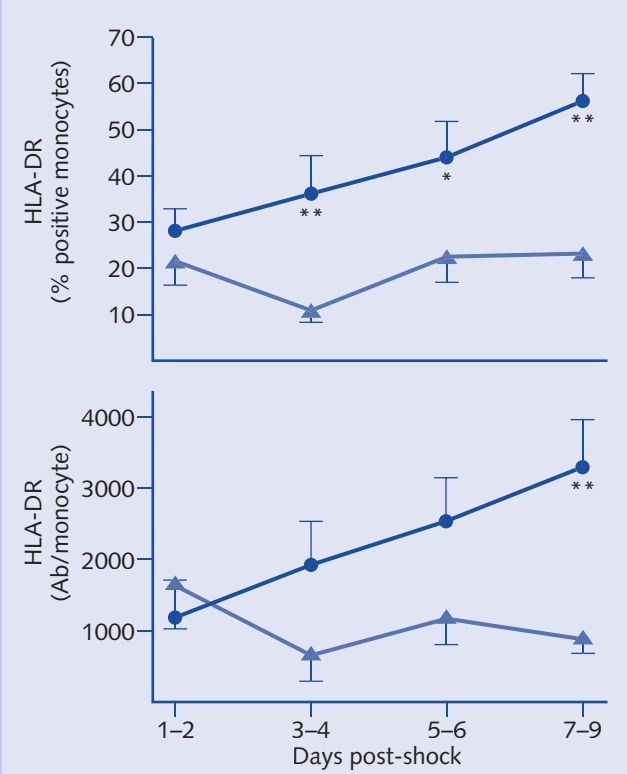
An ongoing study by Monneret et al. has investigated several patients immediately after the onset of shock. mHLA-DR values were >50% in the first hour and subsequently decreased to <20% in both survivors and nonsurvivors, demonstrating that early values may not be informative (personal communication, 2004). Large clinical studies using multiple logistical regression analysis are now

Figure 2. Schematic representation of monocyte HLA-DR expression in patients with septic shock over time.



HLA-DR: human leukocyte antigen type DR.

Figure 3. Changes in HLA-DR expression on monocytes from patients with septic shock. Results are expressed as the mean percentage of monocytes expressing HLA-DR (top) and as the number of antibodies bound per cell (bottom) in patients who survived (n=22, ▲) or died (n=16, ●). The error bars show the standard error (SE). The mean values (±SE) for 54 healthy donors were 90±1% and 15 000±800, respectively.



*p<0.05 vs. nonsurvivors; **p<0.001 vs. nonsurvivors.
Ab: antibodies; HLA-DR: human leukocyte antigen type DR.
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required to assess the potential of mHLA-DR as a marker of immunoparalysis. The inconsistent results observed in the preliminary studies may be due to the lack of standardization in flow cytometry protocols [47], a major limitation in the determination of cell surface markers. Indeed, flow cytometry cannot yet be considered as a standardized tool, and many variables must be taken into account to ensure the technical quality of the results. This is of particular importance when determining mHLA-DR values, as this marker may be rapidly up- or downregulated [46]. A standardized test system has recently been proposed that uses calibrated beads to convert mean fluorescence intensities into the number of antibodies fixed per cell, thus providing an approximation of the number of HLA-DR molecules per monocyte [48]. Use of this method would allow comparison of results from different laboratories, although comparing results expressed as a percentage of HLA-DR-positive monocytes with those expressed as fluorescence intensities showed that while these two parameters are correlated, they may provide different information [49]. This preliminary study in 38 patients with septic shock found that the percentage of HLA-DR-positive monocytes provided better prognostic results than fluorescence intensities (Fig. 3) [5], which was also observed in another study [33]. It is likely that the wide range in levels of HLA-DR expression on monocytes led to the increased variability when results were expressed as number of molecules per cell. For example, a patient with >90% HLA-DR-positive monocytes may possess 15 000–70 000 HLA-DR molecules per cell. The ranges of these two values are quite different: the percentage of HLA-DR-positive monocytes ranges from 0–100%, whereas the number of molecules per cell ranges from 0–70 000. In terms of flow cytometry, it is better to express results as mean fluorescence intensities. However, within the context of immunoparalysis, the percentage of HLA-DR-positive monocytes may provide more information on the functionality of immune cells. It is more valuable to know that the percentage of positive cells is >60% (indicating a good recovery) than to know the exact number of molecules. It remains to be demonstrated which way of expressing mHLA-DR levels best correlates with prognosis.

Monitoring sepsis-induced apoptotic processes

Apoptosis is another promising, albeit little studied, indicator of immunoparalysis. In experimental sepsis, excess apoptosis has been reported to be deleterious [50], while blockade of apoptosis reduced mortality rates [51]. In septic patients who died, Hotchkiss et al. observed lymphocyte apoptosis in

the spleen and a decrease in the number of circulating lymphocytes, contributing to sepsis-induced lymphopenia [52,53]. The same researchers have demonstrated the immunosuppressive properties of apoptotic bodies in a CLP model [54]. Adoptive transfer of apoptotic splenocytes induced immunosuppression by downregulating IFN-γ production and facilitated bacterial dissemination.

Some studies have attempted to monitor apoptosis in septic patients. Using annexin V and flow cytometry analysis on fresh whole blood, Le Tulzo et al. demonstrated that apoptosis rapidly follows the onset of septic shock and leads to profound lymphopenia that is associated with death [55]. Another study showed that expression of the Bcl-2 gene, which encodes an anti-apoptotic protein, was downregulated in critically ill patients at the time they developed nosocomial infection [56]. However, some studies have demonstrated a decreased rate of neutrophil apoptosis in septic patients [57]. These apparently conflicting data remain to be clarified. Based on the results of the initial clinical studies, monitoring of apoptosis deserves further investigation in larger populations to establish its suitability for the early detection of immunoparalysis in septic patients.

Towards a genomic approach

It is likely that the measurement of a single parameter, however accurate, will not be sufficient to characterize the mechanisms that determine the complex and rapidly changing immunological status of patients with sepsis. Consequently, the successful treatment of septic patients may rely on the development of innovative biological tools capable of providing an overview of immune disorders. One promising approach involves post-genomic technologies. Rather than the traditional approach of focusing on one gene or protein at a time, genomic-scale methodologies permit a global view of patterns of coordinated gene expression following exposure to biological stimuli [58]. Defining large-scale gene expression profiles should allow the identification of subsets of genes that function as markers of immunoparalysis or predictors of septic complications and mortality. To date, gene expression profiling has mainly been assessed in different types of cancer. Little work has been devoted to sepsis and there is currently no report available on septic patients. In a preliminary study, Pachot et al. examined the timecourse of expression of 10 cytokine-related genes in peripheral whole blood from septic shock patients and looked for similarities in gene expression patterns between individuals [59]. Combining the expression levels of four genes, up to 95% of the patients with the same outcome showed similarities in mRNA profiling. Importantly, the most useful combination included two genes that were not differentially expressed in survivors and nonsurvivors when interpreted individually, due to overlapping values.

The effect of genetic polymorphisms in sepsis and their putative role in prognosis should also be considered. In addition to the effects of sex hormones and gender on immune responses, epidemiological studies have demonstrated the importance of genetics on the outcome of septic patients [60], illustrating the wide variation in individual responses to bacterial components. Most of this variation between individuals is due to single-nucleotide polymorphisms (SNPs). Correlations have already been established between several SNPs and outcome in sepsis, suggesting that they may be linked with immunoparalysis. However, convincing data on the analytical reliability of DNA microarrays and rigorous clinical trials are required before genetic polymorphisms can be used to monitor septic patients. If positive results are obtained, a crucial step in the development of these methods will be the capacity to analyze such large amounts of information in real time. This might appear to be an impossible challenge, but it is a worthwhile one given that a mortality rate of 50% has been associated with sepsis for the last 25 years.

Conclusion

Taken together, recent clinical and experimental studies indicate that patients with sepsis have features consistent with immunoparalysis. Consequently, upregulation of the host defence system may be a promising therapeutic approach. Several molecules have already been shown to decrease mortality rates and/or the rate of nosocomial infection in preliminary studies [36,44,61–63], although, as these trials did not include large numbers of patients, it is not yet possible to comment on the clinical impact of the results. The assessment of immune functions to identify those patients who may benefit from pro- or anti-inflammatory therapies must be addressed when designing future trials. This has been called "mediator-directed therapy" and is dependent on the ability to measure several immune parameters correctly to establish the presence of immunoparalysis [64,65]. In the opinion of this author, mHLA-DR, IL-10, and apoptosis markers are the most likely candidates, although the imminent post-genomic revolution may reveal further suitable molecules.

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Disclosure

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