Sepsis-induced Innate and Adaptive Immune Suppression

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Abstract: The inflammatory response to sepsis has classically been characterized by an overactive innate immune response. Recent adult and pediatric evidence suggests that the immune response is quite dynamic in this setting, often with endogenous innate and adaptive immunosuppression following the onset of sepsis. Sepsis-induced innate immune dysfunction can include reduction in antigen presenting capacity (as evidenced by decreased monocyte HLA-DR expression) and low pro-inflammatory cytokine production capacity (as evidenced by reduced *ex vivo* LPS-induced tumor necrosis factor-α production). The term immunoparalysis describes a state of severe reduction in these parameters and has been associated with increased risks for the development of nosocomial infection and death in septic adults and children. Intriguing evidence suggests that immunoparalysis may be reversible with beneficial effects on outcomes. Adaptive immune dysfunction has also been reported following the onset of sepsis. Lymphopenia, lymphocyte apoptosis, and skewing toward anti-inflammatory T cell subtypes (such as regulatory T cells) have all been associated with adverse outcomes from sepsis. Without specific testing, most aspects of sepsis-related immune dysfunction are occult. Accordingly, the development of robust immune monitoring and modulation protocols should be a high priority in the battle to improve outcomes from sepsis in critically ill adults and children.

INTRODUCTION

It was classically thought that sepsis represents an over-activation of the immune system with exaggerated release of pro-inflammatory cytokines such as interleukin (IL) – 1β and tumor necrosis factor (TNF)-α. Indeed, the signs and symptoms which define sepsis typically result not from the infection itself, but from the systemic immune response to that infection. To this end, multiple attempts have been made to treat sepsis by decreasing the magnitude of this response through the targeting and neutralization of specific pro-inflammatory mediators [1-7]. Unfortunately, despite promising results in animal models these trials have largely failed to improve outcomes in septic patients. The disparity between the human and animal data may be partly attributable to the fact that humans increasingly *survive* the initial pro-inflammatory phase of septic disease, with many instances of sepsis-related mortality occurring later in the context of multiple organ failure and secondary infection [8, 9]. We now know that the natural history of sepsis in the era of intensive care medicine involves a dynamic immunologic state influenced by both pro- and *anti*-inflammatory forces (Fig. **1**). Cytokines such as IL-10 and transforming growth factor (TGF)-β, both potently anti-inflammatory, likely play as important a role in the pathophysiology of sepsis as their pro-inflammatory counterparts (Table **1**). This review will focus on the compensatory anti-inflammatory response syndrome (CARS) in sepsis including its

quantification and associations with adverse outcomes and will address the innate and adaptive arms of the immune system in turn.

SEPSIS-INDUCED INNATE IMMUNE SUPPRESSION

The bulk of the initial work done to investigate the immunobiology of sepsis centered on the cells and mediators of the innate immune system [10-13]. Innate immune cells are the first cellular responders to invading organisms and are therefore vital to host defense. Cell types including monocytes (and their mature form, tissue macrophages), dendritic cells, and neutrophils recognize pathogens through constitutively expressed receptors including toll-like receptors (TLR) and receptors for the Fc portion of immunoglobulin. Once stimulated, these cells should rapidly and robustly produce pro-inflammatory cytokines. In addition, innate immune cells should be able to recognize and ingest pathogens as well as process and present antigens to members of the adaptive immune system. Monocytes, for example, present antigens on cell-surface major histocompatibility complex (MHC) class II molecules such as human leukocyte antigen (HLA)-DR.

Measurement of monocyte HLA-DR expression was among the first ways that the anti-inflammatory response in critical illness was quantified in the laboratory [10, 11, 14]. To measure HLA-DR expression, blood samples are stained with fluorochrome-tagged antibodies against CD14 (a monocyte cell surface marker) and HLA-DR. Using flow cytometry, the fluorescence of the monocyte anti-HLA-DR antibody can be compared to a negative isotype control. The *percentage of monocytes strongly expressing HLA-DR* can therefore be quantified. Volk *et al.* described monocyte

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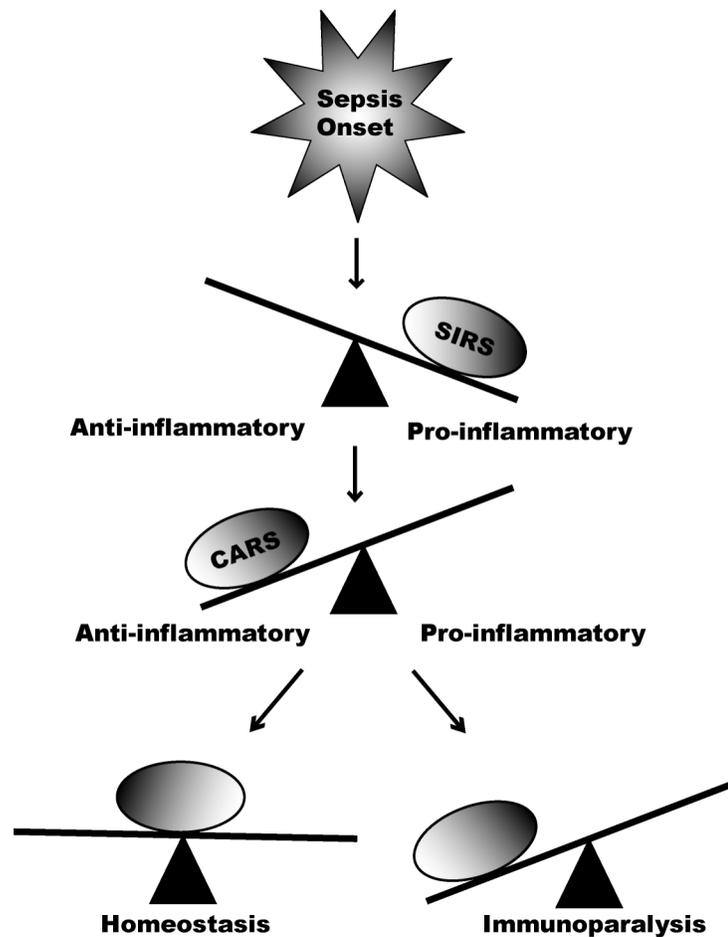E-mail: Mark.Hall@NationwideChildrens.org

Fig. (1). *The dynamic inflammatory response to sepsis.* At the onset of sepsis, patients experience a pro-inflammatory surge known as the systemic inflammatory response syndrome (SIRS). This is typically followed, in a matter of hours, by a compensatory anti-inflammatory response syndrome (CARS), characterized by hyporesponsiveness of the innate immune response. In the subsequent hours to days, the patient should achieve a state of immunological homeostasis. In some patients, however, a prolonged anti-inflammatory phenotype persists. This is known as immunoparalysis.

HLA-DR expression in several cohorts of adults with sepsis and other forms of critical illness in the 1990s [13, 15, 16]. His group showed that monocyte HLA-DR expression was downregulated following the onset of a pro-inflammatory insult, but that mild or transient downregulation was *not* associated with adverse outcomes. Only patients whose monocyte HLA-DR expression fell below 30% for more than 5 days were at increased risk for death [13]. This degree of severe innate immune dysfunction has been termed "immunoparalysis". More recently, Monneret *et al.* showed in 86 adult patients with sepsis that persistently low HLA-DR expression (< 30%) at Day 3-4 of illness was independently associated with increased mortality (OR 6.5; 95%CI 1.6-26) [8]. Similarly, in a study of 20 septic adults Abe *et al.* demonstrated decreased monocyte HLA-DR expression on Day 3 of illness in nonsurvivors (44% in nonsurvivors, 72% in survivors, 92% in healthy controls; p <0.05) [17].

Alternatively, innate immune function can be measured by quantifying the potential of whole blood to produce pro-inflammatory cytokines when stimulated *ex vivo* with lipopolysaccharide (LPS). Whole blood containing normal innate immune cells will produce high levels of TNFα when co-incubated with LPS. Adaptive immune cells, lacking the TLR-4 receptor for LPS, likely contribute little to this acute *ex vivo* cytokine response. This capacity to produce TNFα upon *ex vivo* stimulation of whole blood with LPS has been shown to be reduced in the hours and days following pro-inflammatory stimuli such as sepsis, trauma, and cardiopulmonary bypass in adults and children [18-22]. We have recently shown that the depth and duration of this drop in pro-inflammatory capacity is related to mortality from pediatric multiple organ dysfunction syndrome (MODS) [23].

Unfortunately, standardization of *ex vivo* stimulation techniques has been lacking in the field. There are, therefore, no universally accepted thresholds for definition of immunoparalysis by this method. For example, in our laboratory with our reagents and our sample processing technique a TNFα production threshold of < 200 pg/ml predicts adverse outcomes. Another laboratory using a different type of LPS or incubation procedure may find a different cutoff. The need to develop highly standardized measures of innate immune function is urgent, given the fact that it is not possible to detect immunoparalysis through typical screening laboratory values such as complete blood

Table 1.   Characteristics of the Pro- and Anti-Inflammatory Immune Responses to Sepsis

	Proinflammatory Response	Anti-Inflammatory Response
Characteristic cytokines	TNFα	IL-10
	IL-1β	TGFβ
	IL-6*	IL-1ra
	IL-8	sTNFr
	IL-17	
	IFNγ	
	IL-2	
Innate immune function: phagocytosis, intracellular killing, antigen presentation	Increased	Decreased
Adaptive immune cell types	Th1, Th 17	Th2, Treg

*IL-6 has both pro- and anti-inflammatory properties but is an outstanding marker of the magnitude of the pro-inflammatory response. TNF: tumor necrosis factor, IL: interleukin, TGF: transforming growth factor, IL-1ra: IL-1 receptor antagonist, sTNFr: soluble TNF receptor, IFN: interferon, Th: helper T cell, Treg: regulatory T cell

counts. The variability in currently reported *ex vivo* stimulation procedures makes it difficult to identify thresholds for potential interventions.

Similarly, measurements of monocyte HLA-DR expression may vary based on laboratory techniques including time from blood sampling to antibody staining, the type of anticoagulant used, individual flow cytometer settings, and variations between lots of fluorochromes [24]. Recently, Döcke *et al.* described the use of a technique whereby HLA-DR expression from patient samples is compared to manufacturer-provided standards such that the number of HLA-DR molecules per monocyte can be calculated. With this method, variations attributable to flow cytometers and settings were minimized, though inter-laboratory differences continued to exist due to inconsistent pre-analytic processing and handling of specimens [25]. Clearly, additional work needs to be done to promote uniformity in the diagnosis of sepsis-induced innate immune suppression.

EVIDENCE FOR THE REVERSIBILITY OF SEPSIS-INDUCED INNATE IMMUNE SUPPRESSION

Mounting *in vitro* and *in vivo* evidence suggests that immunoparalysis is reversible. Fumeaux *et al.* were able to induce immunoparalysis in healthy donor monocytes by incubating them with plasma from septic patients. The resultant reduction in HLA-DR expression was significantly improved by the addition of a neutralizing antibody against IL-10 (an anti-inflammatory cytokine) to the cell culture media [26]. Several small case series have suggested that the administration of an immunostimulatory drug can reverse innate immune dysfunction in critically ill adults. Döcke *et al.* gave interferon (IFN)-γ to nine adults with sepsis-induced immunoparalysis and demonstrated normalization of monocyte HLA-DR expression and *ex vivo* LPS-induced TNFα production capacity following treatment [22]. Nierhaus *et al.* showed similar results using granulocyte

macrophage colony-stimulating factor (GM-CSF) [27]. In both case series mortality was improved compared to historical controls. In 2005 Rosenbloom *et al.* performed the first randomized controlled trial of GM-CSF in the setting of adult sepsis [28]. They demonstrated in 33 patients that GM-CSF administration was associated with improved monocyte HLA-DR expression and more rapid resolution of infection compared to placebo. In the pediatric population, Bilgin *et al.* demonstrated a 20% reduction in mortality in a 60-patient randomized controlled trial of GM-CSF for the treatment of sepsis in neutropenic neonates [29]. These latter two trials are notable for the fact that they did not employ *a priori* measurements of innate immune function at the time of enrollment and randomization (with the exception of the neutrophil count in the Bilgin study). It is possible that targeted immunostimulatory therapies aimed at patients with reduced monocyte HLA-DR expression and/or *ex vivo* LPS-induced TNFα production may produce more striking results. Accordingly, Meisel *et al.* recently conducted a prospective, randomized, placebo-controlled clinical trial of GM-CSF in 38 adults with sepsis-induced immunoparalysis, in which severe reduction in monocyte HLA-DR expression was a study entry criterion [30]. In this study, GM-CSF therapy resulted in rapid normalization of monocyte HLA-DR expression and *ex vivo* LPS-induced TNFα production capacity compared to controls. This restoration of innate immune function was associated with reduced durations of mechanical ventilation in the GM-CSF-treated subjects.

SEPSIS-INDUCED ADAPTIVE IMMUNE SUPPRES-SION

The adaptive immune system is comprised of lymphocytes. In contrast to innate immunity, the adaptive immune system promotes the development of highly pathogen-specific effector cells that respond to discrete antigens. Lymphocytes usually rely on members of the innate immune system for antigen processing and

presentation, without which cells of the adaptive immune system typically cannot become activated. Once activated, B lymphocytes mature into antibody secreting plasma cells. Meanwhile, T lymphocytes are grouped by cell surface marker expression into families that perform a variety of biological functions. While CD8+ (cytotoxic) T cells are responsible for directly killing infected cells, CD4+ (helper) T cells produce cytokines to direct the development and activation of other parts of the immune system. These processes are characterized by the development of immunologic memory, such that the response to the first exposure to a particular antigen is slower and less robust than subsequent exposures. In the setting of sepsis, therefore, the adaptive immune system is thought of as being important for the modulation of the *subacute* phase of illness, exerting its effects on the order of *days* after sepsis onset, rather than hours.

CD4+ T cells are notable for their immunomodulatory role and they have traditionally been divided into subgroups of T-helper (Th) cells based on their cytokine release profiles. Th1 cells secrete pro-inflammatory cytokines such as IFNγ while Th2 cells produce anti-inflammatory cytokines such as IL-10 and IL-4 which skew the immune response toward antibody production and away from cell-mediated killing. Th17 cells are a recently elucidated pro-inflammatory subset of helper T cells. They are identified by the production of IL-17 and IL-22, seem to play an important role in autoimmune pathology, and may contribute to the overall pro-inflammatory adaptive immune phenotype in other illness states as well [31].

REGULATORY T CELLS

A fourth family of CD4+ T cells are the regulatory T cells (Treg). This group of cells has the ability to profoundly suppress both the innate and adaptive immune responses by virtue of anti-inflammatory cytokine production and direct, cell contact-mediated inhibition. Among these, "naturally occurring" regulatory T cells develop in the thymus and represent about 5-10% of the circulating T lymphocyte pool in normal individuals. They play an important role in protecting against autoimmunity by suppressing the response to self antigens [32, 33]. Naturally occurring Tregs highly express the cell surface marker CD25, though activated T cells express some CD25 as well which may hinder the recognition of these cells *in vivo* [34]. FoxP3 is a transcription factor important in the development and suppressive activity of naturally occurring Tregs and may aid in their identification [35]. Recently, downregulation of the expression of CD127 (the IL-7 receptor) has also been used to distinguish naturally occurring regulatory T cells from other lymphocytes. CD127 expression negatively correlates with FoxP3, and low expression of CD127 has been found to correlate more closely with suppressive activity than that of CD25 [36, 37].

In addition to naturally occurring regulatory T cells, there exist at least two subsets of CD4+ "inducible" regulatory T cells. These cells are CD25 and FoxP3 negative and have no known specific cell surface markers. While inducible Tregs have been generated *in vitro* under certain culture conditions, their presence *in vivo* can only be inferred through their ability to produce massive amounts of anti-inflammatory

cytokines when stimulated. For example, Tr1 cells produce IL-10 [38-41] and Th3 cells produce TGF-β [42], both in far greater quantities than can be accounted for by Th2 cells alone.

SEPSIS-INDUCED LYMPHOCYTE APOPTOSIS

Several animal studies have demonstrated decreased numbers of lymphocytes and increased lymphocyte apoptosis in response to sepsis [43-47]. Reversal of lymphocyte apoptosis has resulted in improved survival in these models. In 2001, Hotchkiss *et al.* showed reduced numbers of splenic and circulating B and T lymphocytes in adult critically ill patients with sepsis compared to non-septic critically ill patients and trauma patients [48]. These and other data have implicated apoptosis as the source of lymphocyte loss [49]. A subsequent study of 52 adults demonstrated more lymphocyte apoptosis in patients with septic shock compared to controls on Day 1 of illness. In this study, survivors had an increase in lymphocyte count by Day 6, while nonsurvivors continued to have lymphopenia [50]. A similar effect has been seen in children with multiple organ failure. In a study of 113 critically ill pediatric patients, prolonged lymphopenia (an absolute lymphocyte count < 1000 cells/mm^3 for > 7 days) was associated with dramatically increased odds of secondary infection and death [51]. Taken together, these data suggest that lymphopenia represents an important form of adaptive immune suppression in the setting of sepsis, though mechanisms responsible for lymphocyte apoptosis in sepsis remain incompletely understood.

REGULATORY T CELLS AND SEPSIS

Several animal studies have demonstrated increased percentages of CD4$^+$CD25$^+$ Tregs in the setting of sepsis, though data on their impact on outcomes in these models are conflicting. In a study of mice subjected to cecal ligation and puncture (CLP) to induce polymicrobial sepsis, the proportion of CD4+ T cells expressing markers of naturally occurring regulatory T cells was increased compared to control mice [52]. Further, CD4+CD25+ cells taken from septic mice suppressed proliferation of non-regulatory T cells in *ex vivo* assays to a greater extent when compared to Tregs from non-septic mice. Interestingly, depletion of CD4+CD25+ Tregs did not alter mortality in these animals. Similarly, Wisnoski *et al.* in 2007 showed an increase in CD25+ Tregs in mice following CLP [53]. In this study, CD4+ cells from septic mice showed decreased proliferation upon *ex vivo* stimulation which was restored by pre-treatment with anti-CD25 antibodies to deplete the Treg population. Mortality from CLP was again unaffected by Treg depletion. In contrast, Heuer *et al.* demonstrated an *increased* survival and improved bacterial clearance when mice were treated with stimulated Treg cells either before or 6 hours after CLP [54]. Conversely, Scumpia *et al.* showed that blocking the glucocorticoid-induced TNF receptor (GITR), an intervention which should reduce Treg-mediated suppression, prior to CLP increased T cell proliferative capacity as well as pro-inflammatory Th1 cytokine release and *improved* mortality [55]. Reasons for these differences are unclear. One potential confounder is the use of anti-CD25 antibodies for Treg depletion. Because some activated

Table 2.   Common Immune Phenotypes Following Sepsis

	Normal	**CARS**	**Severe Immune Suppression**
Monocyte HLA-DR expression	> 95%	30 – 95%	< 30%
Whole blood *ex vivo* LPS-stimulated TNFα production capacity	+++	++	+
Plasma IL-10	ND	+	++
Lymphocyte count	+++	++	+
Treg percentage	+	++	+++

ND: Not detectable.

T cells also express CD25 it is possible that some activated effector T cells were affected by Treg depletion.

Regulatory T cells have been studied in septic humans as well. In a cohort of 16 adults with septic shock, naturally occurring Tregs were increased compared to other CD4$^+$ T cells in the setting of sepsis-induced immunoparalysis [56]. Specifically, nonsurvivors showed elevated percentages of CD4$^+$CD25$^+$ Tregs 7-10 days after the onset of septic shock compared to survivors. A subsequent study of 54 adult patients showed a significant increase in naturally occurring Tregs at Days 1-2 and Days 3-6 following onset of septic shock in the context of a marked decrease in total CD4+ count and CD4+CD25- effector T cells compared to healthy controls [57]. In other words, the Treg population appeared to remain robust while other T cells disappeared from the circulation. Similarly, a study of 19 adult trauma patients demonstrated decreased numbers of total CD4+ cells with an increase in percentage of CD4+CD25+ Tregs by Day 7 post injury [58]. Tregs isolated from injured patients showed increased suppressive activity, produced less IFNγ (pro-inflammatory) and more IL-4, IL-5, and IL-10 (anti-inflammatory) upon *ex vivo* stimulation than Tregs taken from healthy controls. In these patients, *ex vivo* production of IL-10 and IL-4 by CD4+ T cells isolated on Day 1 of injury was associated with the subsequent development of sepsis.

These data suggest that sepsis or trauma may result in an increase in the relative number and suppressive activity of regulatory T cells. Mechanisms underlying this augmented Treg response are not well understood. One possibility is that regulatory T cells may be less susceptible to sepsis-induced apoptosis or that they may recover more quickly. There is some evidence to suggest that regulatory T cells are less susceptible to dexamethasone-induced lymphocyte apoptosis and T cell receptor-mediated cell death [59, 60]. Perhaps, regulatory T cells respond differently to apoptotic signals compared to effector T cells in a septic environment.

Little is known about the relationship between innate and adaptive immune suppression in septic patients. In the Monneret study, innate immune function as determined by monocyte HLA-DR expression was markedly reduced by the *first day* following disease onset [8]. Survivors began recovering HLA-DR expression by day 7-10, while nonsurvivors, who had an increased percentage of naturally occurring Treg cells by this time, did not. One could hypothesize that a Treg-dominant adaptive immune response may serve to perpetuate the immunoparalyzed phenotype, though this interplay is speculative at this time.

CONCLUSION

There are undoubtedly septic patients who suffer morbidity and mortality as the result of an uncontrolled pro-inflammatory response. We are learning, however, that many patients are dying in the setting of an *anti*-inflammatory immune phenotype. These phenotypes (summarized in Table **2**) can be quantified through relatively easy laboratory measurements, though these tests have not yet found their way into standard practice at most U. S. institutions in some part due to a lack of assay standardization.

Nonetheless, without these assays immunoparalysis is likely to remain occult, despite the availability of promising therapies for its reversal. To that end, further work to define immune monitoring *and* immunomodulatory regimens targeting high-risk septic patients is urgently needed to promote immunologic balance in both the innate and adaptive immune systems.

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