Sepsis:
Staging and Potential Future Therapies

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ABSTRACT

Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection. Variability in pathogenesis and complex pathophysiology often delay diagnosis and create significant challenges for clinical studies in this group of critically ill patients. Mainly for those reasons, there is no therapy approved so far to overcome the underlying immune dysregulation. This book provides an overview about the state of the art of sepsis diagnostics and potential future therapies. Chapter 1 focuses on the immunologic staging of sepsis—the key for successful treatment of the dysregulated hot response. Chapter 2 reveals similarities in the immune response in sepsis and cancer—opening new avenues for novel therapies. Chapter 3 introduces an important modulator of the immune response—the endogenous cannabinoid system and elucidates its role in organ dysfunction in sepsis. Facing the increasing bacterial resistance to classical antibiotics, Chapter 4 discusses two unique mechanisms to treat infection and inflammation in sepsis: iron chelation, and the sphingosine pathway. The authors, all experts in experimental and clinical sepsis research, seek to provide further understanding of the complexities of the immune response as the physiological basis for the development of new therapeutics in sepsis.

KEY WORDS

sepsis, septic shock, trauma, nosocomial infection, immunosuppression, innate immune system, adaptive immune system, immunomodulation, immune dysregulation, flow cytometry, fluorescence-activated cell sorting, INTERCEPT trial, cytokines, pro-inflammatory cytokine, C-reactive protein, t-cell anergy, t-cell exhaustion, microbe-associated molecular patterns (MAMPs), damage-associated molecular patterns (DAMPs), PD-L1, cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), Interleukin-7, tumor necrosis factor-α, NORASEPT trial, RAMSES study, LPS (endotoxin), endocannabinoid system (ECS), inflammation, microcirculation dysfunction, multiple organ dysfunction syndrome (MODS), gastrointestinal barrier function, gastrointestinal motility, acute lung injury, iron chelation, sphingosine, iron hemostasis, reactive oxygen species, oxidative stress, hepcidin antimicrobial peptide (HAMP), intracellular iron

Contents

Introduction

Considerable advances have been made in the understanding of the pathophysiology of sepsis. Sepsis is now defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection. This novel definition focuses on the complex changes within the immune system during sepsis, in contrast to more simplified approaches, such as severe infection and systemic inflammation. From the early phases of sepsis, pro- and anti-inflammatory pathways are activated in parallel. This dysregulation continues even when the initial infectious focus is cleared, potentially resulting in an exhausted, paralyzed immune system.

Today, we have access to a huge repertoire of immune modulating drugs. Of importance for therapeutic success is the exact staging of the immune status of the specific sepsis patient—finding the right lock for the key. It will become necessary for future advances in sepsis diagnostics and treatment to incorporate modern immunological methods, such as flow cytometry protocols into clinical routine. In combination with genetic testing and -omics approaches, we will be able to provide individualized (i.e., personalized) medicine for every patient.

Pro- and anti-inflammatory immune mechanisms are also observed in cancer. Tumor growth and progression is dependent on avoiding immune detection and destruction through the promotion of an immunosuppressive environment. At the same time, cancer-mediated inflammation is necessary to promote growth through tissue remodeling, angiogenesis, and synthesis of growth factors. Those similarities in cancer and sepsis pathophysiology suggest that anti-neoplastic approaches might be useful to treat the dysregulated immune response in sepsis. Indeed, some promising immune modulating therapies originally developed for cancer patients, such as anti-PD1-strategies, are now under consideration for patients with sepsis.

Another interesting mechanism involved in the modulation of the immune response in sepsis is the endocannabinoid system. In the past, this system was studied mostly in relation to its effects on the brain through cannabinoid type 1 receptors. With the discovery of cannabinoid type 2 receptors on immune cells, this endogenous signaling system moved into the focus of studies on local and systemic inflammation, such as sepsis. The anti-inflammatory effects of cannabinoid type 2 receptor upregulation are well established. Furthermore, effects on several organ systems and organ dysfunction in sepsis are described.

Finally, antibiotic therapy, generally considered an irreplaceable initial part of sepsis therapy, faces challenges due to the development of multi-resistant bacterial strains. Novel classes of anti-microbial drugs are needed but not available yet. Therefore, creative approaches are welcomed to overcome the shortage of effective anti-bacterial treatment. Two examples for alternative treatments are iron chelation and sphingosine administration. Both methods are unique because they impact not only bacterial growth but also immune pathways.

CHAPTER 1

Immunologic Staging of Sepsis: Finding the Right Lock for the Key

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1.1 INTRODUCTION

Trauma continues to rank as a leading cause of death among young people (ages 1–46 years), as well as the third leading cause of death across all age groups (Rhee et al., 2014). In addition, the impact of life years lost continues to outpace that of cancer and heart disease. As a result of the advances in medical triage and emergency medicine, hospitalized trauma patients that survive the acute insult are at risk for developing the lethal sequelae of sepsis and septic shock, multiorgan failure, and death.

Despite promising results in animal sepsis models, numerous clinical trials have failed to demonstrate an overall significant impact with immunomodulation via blockade of interleukin (IL)-1, tumor necrosis factor alpha (TNFα), or lipopolysaccharide (LPS) during sepsis. Not only is this attributed to the heterogeneous patient population enrolled but also to the varying degree of immune state and response at the time of agent administration. It is widely established that the septic host initially generates an inflammatory response to an infection. The magnitude of this response is dependent on the number of factors and includes the genetic predisposition to infection, the type infection, and the vigor of the immune response. This inflammatory response is eventually resolved but often the infectious focus is uncleared. The patients that still have an infectious focus, but are not actively mounting a response, can be considered to be immune paralyzed. Thus, the timing of immune modulating agent administration is critical. Administration of an inflammatory suppressing agent (i.e., anti-IL-1 or anti-TNFα) while

the host is immune paralyzed would increase the host susceptibility to nosocomial infections and further decrease the ability to clear the initial infection (Hotchkiss and Karl, 2003). This can be demonstrated by a subanalysis of the INTERSEPT trial (placebo vs 15 mg or 3 mg/kg of TNFα mAb). Here, it was demonstrated that reversal of shock and delayed onset of organ failure among 420 patients with shock when treated with 15 mg/kg of TNFα mAb compared with placebo (Cohen and Carlet, 1996). Similarly, the Interleukin-1 Receptor Antagonist Sepsis Investigator Group failed to demonstrate a reduction in mortality with 72-hour continuous infusion of anakinra (recombinant human IL-1 receptor antagonist), and was terminated early (Opal et al., 1997). Yet, a post hoc analysis by Shakoory et al. (2016) demonstrated that a subgroup of patients with macrophage activation syndrome (fever, disseminated intravascular coagulation, hepatobiliary dysfunction, cytopenias, and hyperferritinemias) had improved 28-day survival (65.4% vs 35.3%) and benefited from anakinra administration compared with placebo. However, these benefits were lost on the overall analysis of patients with and without shock in both studies.

1.2 INNATE AND ADAPTIVE IMMUNE SYSTEMS

In brief, the immune system consists of two components, the innate and adaptive immune arms (Figure 1.1). The innate immune system, otherwise known as the nonspecific or inborn immune system, is very rapidly activated during an infection, and consists of phagocytic cells—monocytes, macrophages, dendritic cells, and neutrophils. Microbes are recognized by these cells using transmembrane pattern recognition receptors (PRR), also known as toll-like receptors (TLR). These receptors are specific for microbial components, or pathogen-associated molecular patterns (PAMP). Upon recognition and binding of PRRs to PAMPs, phagocytosis is initiated.

Macrophages not only phagocytose the pathogens but also process the pathogen-derived proteins into peptides, which are presented by major histocompatibility complex (MHC) molecules on its cell surface. This allows it to communicate with antigen-specific T lymphocytes, a component of the adaptive or acquired immune system. The adaptive immune system consists of humoral and cell-mediated immunity. Humoral immunity involves the maturation of B lymphocytes into plasma cells, responsible for antibody production. T lymphocytes, the major component of cell-mediated immunity, consist of CD4 and CD8 populations. CD4 T cells, also known as helper T cells, promote the immune response when activated by cells of the innate immune system via MHC class II antigens. CD8 T cells, also known as cytotoxic T cells, recognize MHC class I antigens and eliminate infected or tumorigenic host cells.

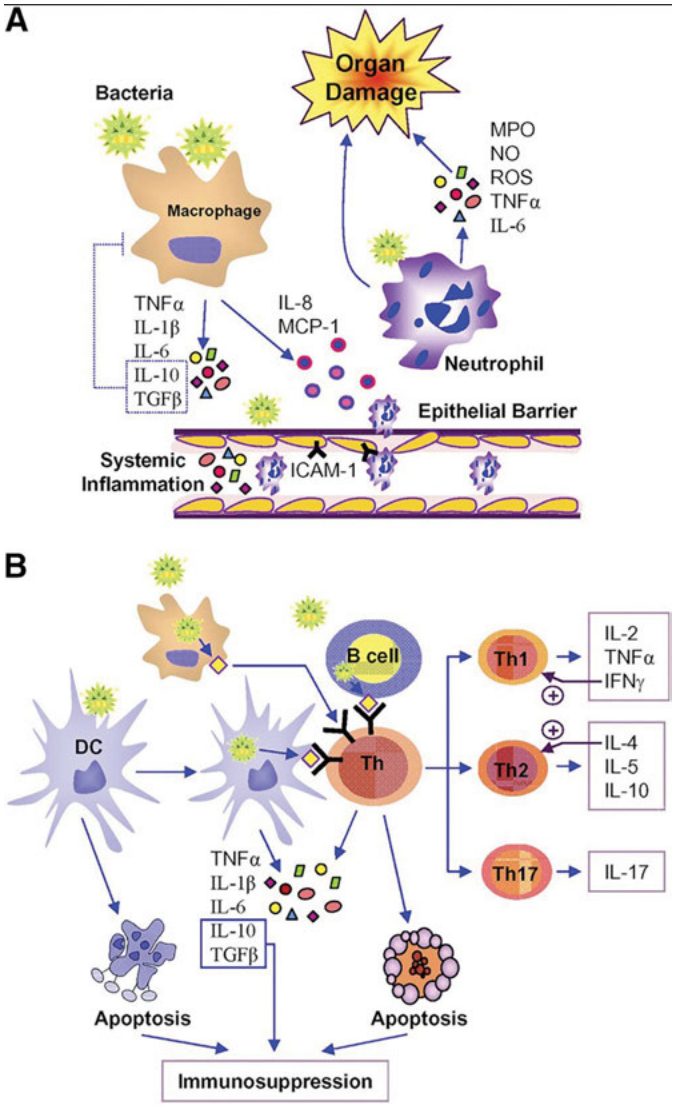

FIGURE 1.1: Responses of the innate and adaptive immune arms to sepsis. A) Surveilling macrophages and neutrophils respond by engulfing bacteria and the secretion of inflammatory mediators. Excessive production of inflammatory mediators can result in organ damage. B) Dendritic cells and lymphocytes undergo apoptosis during the early stages of sepsis. The apoptosis process produces increased generation of anti-inflammatory cytokines (TGF-β and IL-10) and can result in immune suppression. Remaining T lymphocytes continue to play a reduced role in the bacterial removal (Th1), immune resolution (Th2) and neutrophil trafficking (Th17). Figure reprinted from Monowar et al. (2013). Used with permission from John Wiley and Sons.

Currently, the status of the innate and adaptive systems can be assessed by the complete blood count (CBC) with cell differential. In some settings, a C-reactive protein (CRP) level will also be analyzed along with the CBC with differential.

The CBC quantifies the leukocyte numbers, the hemoglobin and hematocrit concentrations, and the platelet count. With the addition of the cell differential, the percentage of neutrophils, monocytes, lymphocytes, eosinophils, basophils, and the presence of any premature neutrophils or "bands" can be determined. The CBC and differential are typically completed by an automated cell counter and only requires minutes to perform. The CBC and differential cannot determine immune differentiation, activation, and suppression states in a septic patient. CBC lacks the sensitivity necessary to quantify immune status. To increase the sensitivity, additional tests are often ordered, including the CRP.

CRP is an acute phase biomarker commonly used to estimate inflammation within the human body. It is often used to predict early infection prior to clinical appearance. Although CRP is an accurate marker of inflammation, many other conditions, including autoimmune disease and sterile burn injury, will also elevate the CRP. Therefore, CRP does not have high sensitivity or specificity. The limitations of the CBC/differential and CRP, coupled by the complexity of immune system dysregulation, suggest that another approach is needed to assess the immune status of critically ill trauma patients.

1.3 DETERMINING THE IMMUNE STATUS OF PATIENTS WITH FLOW CYTOMETRY

Flow cytometry is an impedance-based method of cellular analysis from tissue or fluid samples. Fluorescence-activated cell sorting (FACS), a form of flow cytometry developed and coined by Dr. Herzenberg, characterizes samples one cell at a time, based on its light scatter pattern and fluorescence (Julius et al., 1972). In addition, use of fluorescent-labeled antibody probes to specific cell surface markers improves cellular characterization.

One advantage of flow cytometers is the sample volume required for analysis. For adequate sampling, 10^6 events are required, and sample volumes range from 100 to 500 μl. Thus, a typical patient blood collection tube can be used for multiple analyses.

In patients with acute pancreatitis complicated by organ dysfunction, Oiva et al. characterized, with the use of phosphospecific whole blood flow cytometry, the activated signaling pathways of peripheral leukocytes (Oiva et al., 2010). There was a decrease in NFκB, ERK1/2, and STAT1 phosphorylation levels in patients compared with healthy controls, whereas STAT6

and p38 activation increased, favoring T_h2 differentiation of lymphocytes. The depiction of the immune state during pancreatitis has implications in sepsis, despite the limitations of the study, which only evaluated circulating leukocyte and excluded tissue leukocytes from the site of septic insult.

Flow cytometry has further utility in characterizing and enumerating leukocyte phenotypes, activation, function, and cytokine production from the blood, septic site, and lymphoid tissue. The ratio of neutrophils to monocytes and CD4+ T cells to CD8+ T cells changes during the acute and subacute phases of sepsis and allows for more accurate distinction of the temporal course of sepsis. Surface markers, CD25, CD44, CD69, and CD71, may be assessed for activated T cells, whereas the absence of these markers but presence of CD62L reveal inactivated T cells (Shipkova and Wieland, 2012; De Rosa et al., 2001).

The C5a peptide, a component of the complement system, is extensively involved in the innate immune system, with chemotactic effects on neutrophils and monocytes via the C5a receptor (CD88). Among neutrophils, C5a upregulates adhesion molecules responsible for adhesion to endothelial cells and migration (Foreman et al., 1996). Although antagonism of CD88 or neutralization of C5a was protective in animal models of sepsis and endotoxemia, measurements of neutrophil CD88 levels by flow cytometry were found to be significantly depressed among patients with sepsis or septic shock (Furebring et al., 2002; Czermak et al., 1999). Furthermore, patients with multiple traumatic injuries were found to have significantly reduced leukocyte CD88 expression from admission to 10 days after injury (Amara et al., 2010). Future endeavors to define the rate in recovery of CD88 expression may aid in mapping out the proinflammatory and immunosuppressive phases of sepsis.

The CD64 surface receptor on neutrophils has been reported as a useful marker in sepsis. Resting neutrophils have low expression of CD64, but there is increased expression among activated neutrophils, in parallel to the degree of the inflammatory response (van der Meer et al., 2007). Unlike neutrophil CD11b expression, CD64 expression is stable at room temperature for more than 24 hours, making it suitable as a test in a clinical laboratory. In the pediatric population, it has been demonstrated to be a more sensitive predictor of pediatric sepsis than serum C-reactive protein levels, a test commonly used to gauge the level of inflammation (Groselj-Grenc et al., 2009). However, meta-analysis of 17 studies, comprising 3478 neonates demonstrated a low sensitivity and specificity of 77% and 74%, respectively (Shi et al., 2016). Similarly, a meta-analysis of eight studies, comprising 1986 septic adult patients, reported a pooled sensitivity and specificity of 76% and 85% (Wang et al., 2015). Due to the complexity of sepsis, serum neutrophil CD64 levels alone are likely inaccurate in predicting severity of sepsis. In addition, CD64 expression may be elevated in both bacterial and viral infections, and

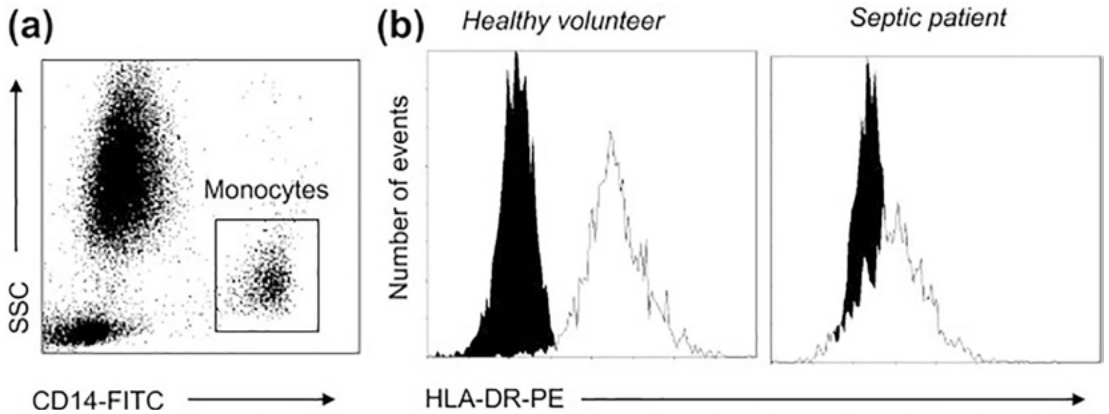

FIGURE 1.2: Example for the use of FACS to stage sepsis. Monocytic HLA-DR expression can be used to gauge immune suppression of a septic patient. A) The monocyte population is selected by the granularity (SSC) and surface marker (CD14). B) Healthy patient expression of HLA-DR that depicts an immune system able to effectively respond to a bacterial infection. C) Septic patient expression of HLA-DR indicating potential exhaustion of the immune system. Reprinted under CC-BY license from Biomed Central.

differentiation has not been established yet. However, in combination with other biomarkers, such as monocytic human leukocyte antigen-D-related (HLA-DR) expression, the accuracy of staging sepsis severity is improved (Juskewitch et al., 2015).

Monocytic HLA-DR marker is upregulated during monocyte activation and allows antigen presentation to T cells. Although other markers are also upregulated during antigen presentation, reduced levels of HLA-DR have been implicated during sepsis and immunoparalysis (Figure 1.2), reflecting a reduced ability to mount an appropriate inflammatory reaction during a secondary insult as well as in antigen presentation (Leentjens et al., 2013). Landelle et al. (2010) associated the reduction in HLA-DR as a biomarker for the development of nosocomial infections. Furthermore, a prospective observational study by Drewry et al. (2016), although underpowered, reported that a median decrease of 934 HLA-DR antibodies/cell between days 1–2 and 3–4 preceded the development of secondary infections.

The adaptive immune system may be assessed for immunosuppression by measuring the T cell response during sepsis. As previously mentioned, absence of surface markers CD25, CD44, CD69, and CD71, and presence of CD62L reflect inactivated T cells with a shift toward Th2 profile and apoptosis (Shipkova and Wieland, 2012; De Rosa et al., 2001). T-cell anergy, a state in which T cells fail to respond to antigenic stimuli, is mediated by regulatory T cells and is detrimental to the septic host (Venet et al., 2009). A decrease in T-cell population

with a predisposition for apoptosis, as well as a decrease in the diversity of the T cell repertoire, may be observed on flow cytometry (Hotchkiss RS and Nicholson, 2006; Nikolich-Zugich et al., 2004). In a prospective, observational study of adult septic patients, decreased T-cell receptor diversity, along with increased population of regulatory CD4+CD25+ T cells was associated with increased likelihood of mortality and developing nosocomial infections (Venet et al., 2013). Stratification of risk for nosocomial infections was strengthened by combining three markers of immune status—neutrophil CD88, monocyte HLA-DR, and percentage of T regulatory cells (Conway et al., 2013). The prospective, multicenter observational Immune Failure in Critical Therapy (INFECT) Study is accruing patients to validate these biomarkers in identifying critically ill patients who develop nosocomial infections (Conway et al., 2016).

1.4 CONCLUSION

The utility of flow cytometry continues to expand, as studies continue to examine the link between biomarkers and sepsis complications. Promising results from the measurement of HLA-DR to stratify for administration of granulocyte-macrophage colony-stimulating factor in critically ill patients may pave the way for future clinical trials to adopt this strategy to more accurately and appropriately target deficits in immune function (Meisel et al., 2009). Previous clinical trials with immunomodulation have been largely ineffective. As the technology continues to improve and our database of model biomarkers grows, it will become necessary for future trials to incorporate flow cytometry protocols to effectively treat patients, as well as to prevent further harm. Additionally, once the ability is gained to stage sepsis, then immune modulating approaches will need to be innovated and tested.

CHAPTER 2

Sepsis and Cancer: Similarities in the Immune Response and Treatment with Novel Therapies

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2.1 INTRODUCTION

According to the Center for Disease Control (Deaths and Mortality), cancer is the second leading cause of mortality in the United States, responsible for over 500,000 deaths in 2013 (Deaths and Mortality). Similarly, sepsis affects over 750,000 hospitalized patients and is the leading cause of in-hospital mortality (Liu et al., 2014). Combined, these two disease processes represent an enormous burden in patient morbidity, mortality, and health care expenditure. Sepsis and cancer also share many similarities in their interactions within the host immune system; thus, these two have common therapeutic targets and potential treatment regimen. The following discussion highlights the pathophysiology of cancer and sepsis as it relates to the immune system. Novel therapies and current clinical trials are also addressed in this chapter.

2.2 SHARED MECHANISMS IN INFLAMMATION AND IMMUNOSUPPRESSION

2.2.1 Mechanisms of Immunomodulation

The innate and adaptive immune responses play a vital role in resisting infectious pathogens and subsequent imbalances in tissue homeostasis (Matzinger, 2002; Medzhitov, 2008). The innate

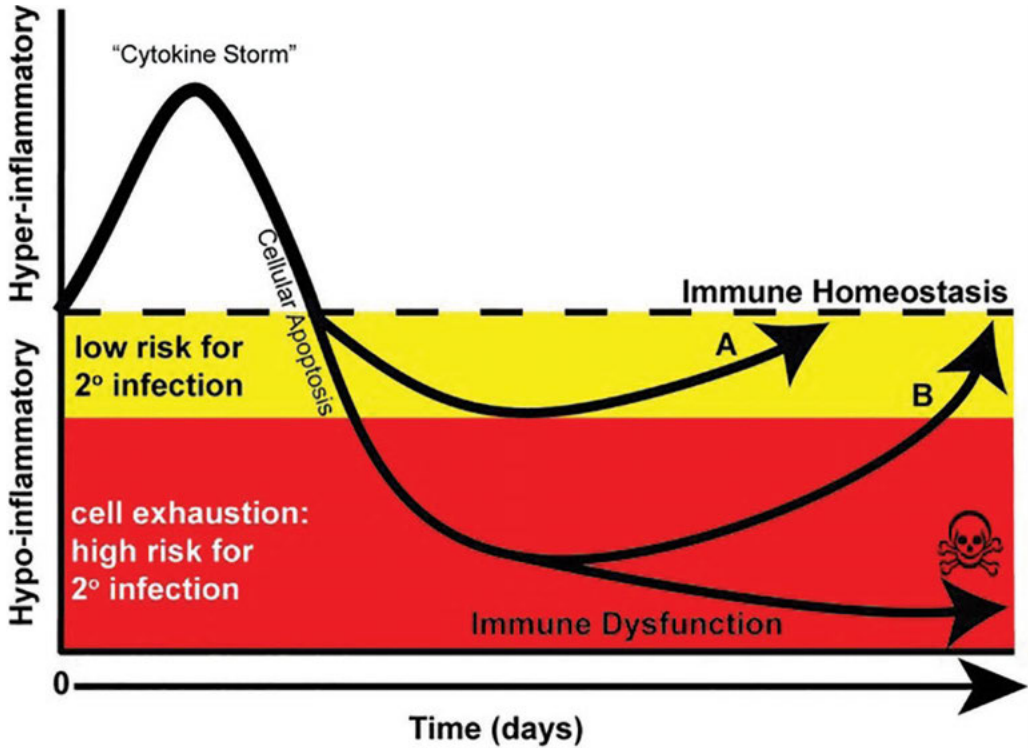

FIGURE 2.1: Temporal changes to the immune system during sepsis. In response to an infection, the immune system mounts a pre-dominant pro-inflammatory response. To check the inflammatory response, pro-resolving mediators are produced. Additionally, leukocytes may undergo apoptosis or become unresponsive. This may allow for an anti-inflammatory or exhausted state to prevail, resulting in a host inability to clear the existing or nosocomial pathogens. This immune suppressed state likely contributes to elevated ensuing morbidity and mortality. Figure reprinted from Boomer et al. (2013). Used with permission of Taylor & Francis.

response, also referred to as the nonspecific immune system, generates a universal response against offending agents but does not adjust itself to the pathogen itself. This is triggered by microbe-associated molecular patterns (MAMPs) and host mediators released by damaged tissues via damage-associated molecular patterns (DAMPs). The adaptive response conversely generates a specific response to the epitopes presented by the offending agent.

Initially, sepsis is characterized as an aggressive inflammatory state (Figure 2.1). This heightened response is comprised of a deluge of proinflammatory cytokines which mediate tissue injury, leading to patient morbidity and mortality (Hotchkiss and Karl, 2003). Biffl et al. (1996) first reported a delay in neutrophil apoptosis with IL-6 incubation, which resulted in more superoxide

production than naïve neutrophils. This may be a mechanism of how the inflammatory milieu contributes to tissue injury. Furthermore, inflammatory cytokines granulocyte-macrophage colony stimulating factor (GM-CSF), tumor necrosis factor α (TNFα), and interferon γ (IFNγ) produced by activated natural killer cells have also been demonstrated to rescue neutrophils from apoptosis and retain its function (Bhatnagar et al., 2010). As the septic response persists, however, the proinflammatory condition subsides and allows for an anti-inflammatory or exhausted state to prevail, resulting in a host inability to clear the existing or nosocomial pathogens (Oberholzer et al., 2001). The immunosuppressive state is arguably as detrimental to the patient as its counterpart and may contribute to elevated ensuing morbidity and mortality (Boomer et al., 2011).

From an oncologic perspective, tumor growth and progression is dependent on avoiding immune detection and destruction through the promotion of an immunosuppressive environment (Grivennikov et al., 2010). At the same time, cancer-mediated inflammation is necessary to promote growth through tissue remodeling, angiogenesis, and synthesis of growth factors (Schetter et al., 2010). This fact was first discovered by Coley in the 1890s, when injection of dead bacterial cultures into patients resulted in a febrile response along with tumor regression (Nauts et al., 1953). Since then, shared pathways in immunologic dysfunction and immune evasion have been studied in both sepsis and cancer.

2.2.2 T-Cell Anergy

Anergy is defined as a state in which T cells fail to respond to antigens. It serves as a key regulatory process in the development of self-tolerance and prevention of autoimmunity. However, when present in a septic host, defective T-cell proliferation and secretion of cytokines IL-2 and TNF correlate with sepsis mortality (Heidecke et al., 1999). After trauma and burns, host T cells demonstrate a predilection to apoptosis, with the remainder becoming anergic (Pellegrini et al., 2000). Monocytes and macrophages also demonstrate increased susceptibility to apoptosis as a result of imbalances in immunomodulatory cytokines, further mitigating cell-mediated immunity during sepsis (Voll et al., 1997). Although the molecular pathway of T-cell anergy is still being investigated, its parallels in tumor-mediated immune evasion have been well documented (Crespo et al., 2013).

2.2.3 T-Cell Exhaustion

Chronic antigenic overstimulation, mediated by DAMPs and MAMPs in cancer and sepsis, may generate a state of relative *T-cell exhaustion* (Hotchkiss and Opal, 2010). T-cell exhaustion was first described in murine models as dysfunction and depletion of antigen-specific T cells

during chronic viral infection (Virgin et al., 2009). It has since been demonstrated in various bacterial infections, human immunodeficiency virus (HIV), sepsis, and cancer (Wherry, 2011). Characteristics of T-cell exhaustion include downregulation of proinflammatory cytokines, such as IL-2, TNF, and IFNγ; as well as a diminished proliferation capability (Boomer et al., 2011). The severity of cytotoxic and helper T-cell exhaustion is correlated with lack of infection and tumor control.

T-cell exhaustion is mediated through several mechanisms, including inhibitory signaling cascades, immunosuppressive cytokines, such as IL-10, and relative proliferation of T-regulatory cell population (Goldszmid et al., 2014). Programmed cell death 1 (PD-1) and its ligand, programmed death ligand-1 (PD-L1), are key factors in a major inhibitory pathway whose expression is increased during sepsis and T-cell exhaustion (Spec et al., 2016). Studies have noted that when PD-1 activity is inhibited, virus-specific $CD8^+$ T-cell response is improved, resulting in decreased viral load and improved survival (Goldszmid et al., 2014). Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) is another ligand expressed by T cells during chronic infection and inflammation. CTLA-4 acts primarily by binding B7 with greater affinity than its intended ligand, CD28, which is expressed on T cells (Thompson and Allison, 1997). Naturally, when CTLA-4 is inhibited, T-cell function is recovered (Goldszmid et al., 2014). The aforementioned molecules, PD-1, PD-L1, and CTLA-4, represent key mediators of negative regulatory pathways that have garnered therapeutic interest in both sepsis and cancer research.

T-cell exhaustion is also mediated by the response of natural killer (NK) cells. In chronic viral infections, such as HIV, NK cells demonstrate changes in cytokine production and cytotoxic function that contribute to T-cell dysfunction (Alter et al., 2010). In addition, the exhaustion and depletion of γδ T cells, which is responsible for inducing maturation in dendritic cells and killing tumor cells, is of therapeutic interest in cancer patients. Investigations in the prevention of T-cell exhaustion by targeting NK cells and γδ T cells may be high yield in addressing sepsis and cancer immunotherapy.

2.3 PROGRAMMED CELL DEATH 1 (PD-1, CD279)

2.3.1 The Role of PD-1 in Cancer

Dong et al. (1999, 2002) first postulated that PD-1 and its ligand, PD-L1, played a role in tumor cell proliferation through immune evasion. PD-L1 levels were found to be upregulated in various solid organ tumors, including lung, ovary, skin, and colon cancers (Dong et al., 1999).