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Immune Cell Dysfunction as a Consequence of Severe Sepsis

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1. Introduction

The pathophysiology of severe sepsis is unique among immunological conditions in that the immune system is ultimately the root cause of the disease, and yet in certain aspects it is the immune system that suffers the most severe after effects following resolution of disease. During the acute phase of the disease, unchecked activation of the immune system results in severe physiological stress, which can lead to multi-organ dysfunction (MODS) and death (G. P. Patel et al., 2003). However, both during the acute phase and following recovery from severe sepsis, the immune system can suffer from significant deficiencies in activation and effector function that can render the patient susceptible to secondary, nosocomial and opportunistic infections (Reddy et al., 2001). This immune suppression is most readily apparent in regards to cellular responses, as both innate and adaptive immune cells exhibit deficiencies in effector responses following the onset of severe sepsis, both *in vitro* and *in vivo*. In certain cases, these deficiencies appear tied to the overstimulation of immune responses during the acute phase of the disease; for example, as macrophages become unresponsive to further stimulation with lipopolysaccharide (LPS) despite Gram-negative organisms being the (one of many possible) root cause(s) of the onset of sepsis (Fujihara et al., 2003). These phenomena may be considered an instance of immune tolerance as a consequence of overstimulation (e.g. "LPS tolerance" in macrophages both *in vivo* and *in vitro*). In addition, many instances of cellular dysfunction persist in survivors of severe sepsis long after the resolution of the acute phase of the disease; these cellular deficiencies are correlated in experimental models of sepsis, and play a key role in the development of post-septic immunosuppression.

Cellular dysfunction following severe sepsis proceeds from the intense inflammatory response triggered by exposure to large quantities of microbes, microbial products, and/or dead and dying cells and tissues. Suppression of activation and effector function of immune cells can be mediated through negative regulation of signalling pathways involved in the sensing of microbes and dead tissues, via direct suppression of immune responses through the signalling activity of anti-inflammatory cytokines, and in the long term, via modifications to the regulation of gene expression necessary for proper activation and effector function of immune cells. The end result of these phenomena is an immune system that lacks the ability to properly mount directed inflammatory responses in the context of secondary infections and other pathologies (such as cancer). The observed cellular

dysfunction following the initial onset of sepsis can be loosely grouped into two distinct categories: acute deficiencies in the context of the systemic inflammatory response syndrome (SIRS), and chronic deficiencies in the context of the compensatory anti-inflammatory response syndrome (CARS) and beyond (N. S. Ward et al., 2008).

1.1 Pathophysiology of SIRS and CARS

The initial onset of severe sepsis can be due to numerous factors, many of which are derived from infectious agents but are not necessarily of infectious origin. For example, LPS derived from Gram-negative bacterial cell walls is a potent initiator of the septic response through its ability to stimulate large amounts of proinflammatory cytokines through Toll-like receptor (TLR)-4 signalling pathways (Zhang & Ghosh, 2000). Additionally, Gram-positive cell wall components (e.g. lipoteichoic acid) can signal through TLR2, initiating many of the same proinflammatory responses as LPS stimulation (Henneke et al., 2008). Shared components of both Gram-negative and –positive bacteria are the presence of unmethylated CpG motifs within the bacterial genome; these CpG motifs can be sensed by TLR9 (Wagner, 2002). Several types of RNA species, especially double-stranded RNA (dsRNA) from viruses, and intracellular RNA released from necrotic host cells (Cavassani et al., 2008), can activate TLR3 (Sen & Sarkar, 2005) as well as intracellular dsRNA sensors such as RIG-I and MDA-5 (Takeuchi & Akira, 2008). Additionally, bacterial superantigens (SAgs) can activate peripheral T cells in a non-specific, polyclonal manner, leading to the undirected production of proinflammatory cytokines and the development of sepsis (Ferry et al., 2005; Fraser & Proft, 2008). Despite the disparity in the instigating agent(s), the pathophysiology of the disease follows a biphasic progression, based on the generalized immune responses to the original stimuli and the subsequent "cytokine storm," respectively.

The systemic inflammatory response syndrome (SIRS) is defined clinically as the presence of two of the following four symptoms: hyper/hypothermia, tachycardia, tachypnea and leukocytosis/leukopenia with exaggerated neutrophils (>10% of peripheral white blood cells) (Conference:, 1992). From an immunological perspective, SIRS is a result of the exaggerated production of proinflammatory chemokines and cytokines by large numbers of activated immune cells. For example, early inflammatory mediators such as Tumor Necrosis Factor-alpha (TNFα), Interleukin-1 beta (IL-1β), IL-6 and IL-8 (CXCL8) are responsible for the variation in body temperature, breathing rate and peripheral white blood cell count, and the overproduction of these mediators can result in further exacerbation of inflammatory responses as well as immune cell apoptosis (Unsinger, McDonough, et al., 2009). Overproduction of proinflammatory chemokines and cytokines by the innate immune system (e.g. macrophages, granulocytes and dendritic cells) can feed back on the adaptive immune system, resulting in amplification of SIRS through production of type I (Kelly-Scumpia et al., 2010) and type II interferons (IFN) (Romero et al., 2010; Silva & Cohen, 1992) and T-cell derived cytokines such as IL-17(Flierl et al., 2008). The "cytokine storm" of SIRS is multifaceted, and reflects the multiple possible instigating agents and the multiple immune cell types participating in the response.

The cytokine storm of SIRS results in morbidity and mortality through the stresses caused by the unchecked inflammation on the host's physiology. For example, SIRS results in a massive apoptotic/necrotic cell death event observable in primary and secondary lymphoid tissues, as well as in the peripheral blood (Wesche-Soldato et al., 2005). This widespread cell death has two major outcomes. First, by significantly reducing the number of available leukocytes, this cytokine-storm induced cell death leaves the patient susceptible to

secondary infections (C. Oberholzer et al., 2001). Secondly, cell components released by dying cells (especially nucleic acids and mitochondrial proteins) can further amplify the cytokine storm through stimulation of TLRs on remaining immune cells – this is especially true in the case of necrosis (Cavassani et al., 2008). Extreme variations in body temperature, heart rate and breathing rate are also mediated by the unchecked production of proinflammatory chemokines and cytokines (Sriskandan & Altmann, 2008). Ultimately, these physiological stresses, combined with adverse coagulation events and damage to the vasculature, can result in SIRS-induced mortality.

The initiation of SIRS is ultimately a result of immune cell activation; as a result, the immune system will often attempt to overcompensate for the overproduction of proinflammatory chemokines and cytokines by upregulating anti-inflammatory mediators. These anti-inflammatory mediators can be immunosuppressive cytokines, such as IL-10 or transforming growth factor-beta (TGFβ), or immune-deviating cytokines, such as T-helper type 2 cytokines (such as IL-4 and IL-13) which attempt to counteract the largely T-helper type 1 response initiated by SIRS cytokines (such as IL-12 and IFNγ) (Adib-Conquy & Cavaillon, 2009). Unfortunately, these compensatory mechanisms often fail to restrict the damaging effects of the cytokine storm of SIRS; instead, they often render those leukocytes that survive the initial apoptotic/necrotic event unable to respond to further infectious stimuli. This is especially dangerous at timepoints proximal to the onset of severe sepsis, as it renders hospitalized patients (undergoing treatment for SIRS) at risk for developing nonsocomial infections. Recent epidemiological studies also suggest that the CARS response may persist in some form for days, months and years following the resolution of severe sepsis and discharge from hospital care, as the both the survival rates and quality of life of survivors of severe sepsis are significantly reduced when compared to the healthy age-matched population (Perl et al., 1995; Quartin et al., 1997).

Cellular immune dysfunction as a result of severe sepsis can therefore be further conceptualized as three distinct phases, all following the initial onset of SIRS due to overstimulation of the immune system. In the first phase, anti-microbial responses are blunted due to "exhaustion" of the inflammatory responses of cells responding to the septic insult. In the second phase, the upregulation of anti-inflammatory responses by the immune system, in an attempt to regulate SIRS, renders the immune system unable to deal with secondary infections; this can be thought of as early-phase CARS, or compensatory immunosuppression. In the third phase, immune cells that have survived severe sepsis retain deficiencies in activation and effector function even after the resolution of disease; this can be thought of as late-phase CARS or post-septic immunosuppression. These last two phases occur as a direct response to cytokine storm of CARS, and are defined loosely based on their proximity to the initiation of severe sepsis. In all three cases, the phenotypic responses of the human patient or animal model (susceptibility to secondary, nosocomial and opportunistic infections) follows in part from the inability of the cells of the immune system to properly respond to challenges following sepsis. The specific mechanisms underlying the immunoparalysis observed in survivors of severe sepsis are specific to both the cell type and the phase of disease progression, but all ultimately deal with deficiencies in the activation, differentiation and effector function of post-septic immune cells.

1.2 Immune dysfunction in sepsis – clinical and experimental outcomes

Epidemiologically, immune suppression following sepsis manifests as a significant decrease in the long-term survival of patients recovered from severe sepsis; this decrease is seen years

after the resolution of the inflammatory episode, as compared to the healthy age-matched population. Additionally, patients who do survive severe sepsis report lower quality of life scores as compared to healthy control populations (Winters et al., 2010). Of the patients that succumb to increased morbidity and mortality following sepsis, there are numerous infectious and non-infectious comorbidities that are correlated with decreased life expectancy. For example, opportunistic infections are commonly associated with patients recovering from septic shock (Benjamim et al., 2004). The long-term decrease in life expectancy observed in patients post-sepsis is described in epidemiological studies, and the underlying etiology of this post-septic mortality is less clear; however, the increase in mortality observed in post-septic patients appears unreleated to associated comorbidities, and is a direct result of the severity of the initial septic episode (Quartin et al., 1997).

Experimentally, post-septic immunosuppression can be studied by utilizing "two-hit" models of disease, whereby experimental animals are subjected to severe sepsis and then challenged with a secondary infection or immunity-stimulating event (such as solid tumor challenge). These models have been critical for both the identification of long term immunosuppression in survivors of severe sepsis, as well as for discerning the underlying mechanisms of cellular immune dysfunction following sepsis. Classical "two-hit" models often utilize a sepsis-triggering effect (such as high dose LPS injection or surgical induction of sepsis, also known as cecal ligation and puncture, CLP) in combination with airway challenges with opportunistic micro-organisms. The use of the lung microenvironment to study secondary infections and post-septic immunosuppression is of particular importance. By utilizing secondary infections that originate in the lung, researchers can study the immune environment in the lung during sepsis, where immune responses can both initiate septic shock and exacerbate deleterious outcomes (such as in the case of acute respiratory distress syndrome) (Hudson et al., 1995).

Experimental models of "two-hit" post-septic immunosuppression often utilize opportunistic pathogens for the second challenge, as healthy animals (untreated, or subjected to sham surgery as a control) would be expected to clear the microorganism without difficulty. One classic example of such an opportunistic pathogen is the fungi *Aspergillus fumigatus*, whose infectious conidia are ubiquitous in the environment and are normally controlled by a fully functioning immune system (Walsh et al., 2005). In immunocompromised individuals, these condidia can germinate in the lung and result in invasive infection. In mice, *A. fumigatus* airway challenge results in little discernible pathology, with the conidia unable to germinate prior to clearance by innate immune cells with phagocytotic activity (such as macrophages and neutrophils). In contrast, mice that have recovered from severe sepsis are unable to generate protective immune responses to *A fumigatus* conidia; these animals ultimately succumb to invasive aspergillosis, characterized by tissue damage mediated by hyphal outgrowth in the lungs (Benjamim et al., 2003). Deficiencies in cellular immune responses in post-septic animals are directly responsible for the susceptibility of post-septic animals to *A. fumigatus*-induced mortality. In addition to fungal challenge, other microorganisms are utilized to study post-septic immunosuppression in murine models. Secondary bacterial challenges are especially useful for modelling nosocomial infections in human patients; susceptibility to secondary infections has been identified in mouse models using *Pseudomonas aeruginosa* or *Listeria monocytogenes,* among others (Delano et al., 2011). Post-septic animals are also more susceptible to the implantation of solid tumors, as tumor size is increased in post-septic animals as compared to controls(Cavassani et al., 2010).

Impaired immune cell function plays a key role in mediating post-septic immunosuppression in all the experimental models discussed previously. In addition, these same deficiencies in immune cell function underlie the susceptibility of human patients to secondary, nosocomial and opportunistic infections following the onset of severe sepsis. Cell populations within the innate and adaptive immune system exhibit distinct phenotypes that mediate the different phases of post-septic immunosuppression, and the interplay between these various celluar deficiencies results in the susceptibility to infection observed in both human patients and animal models.

2. Immune cell dysfunction during SIRS

Despite immune activation being the root cause of severe sepsis, the cytokine storm associated with acute inflammation also feeds back on peripheral immune cells to mediate immunosuppression. This immunosuppression is mediated through two general mechanisms. First, widespread apoptosis of peripheral leukocytes limits the population of immune cells (and immune cell progenitors) available to respond to secondary infections. Second, overstimulation of surviving leukocytes - due to exposure to large amounts of microbial products, apoptotic/necrotic cell products and/or chemokines & cytokines – results in a refractory response to secondary stimulation, as is the case with a concurrent nosocomial infection during severe sepsis. The combination of these two factors results in susceptibility to secondary infections proximal to the onset of sepsis. In human patients, this response manifests as nosocomial infections that present soon after the admittance of the patient to emergency/intensive care; in animal models, this early immunosuppression is modelled with secondary infections early after the onset of sepsis (24-72 hours following induction).

The physiological stress caused by the cytokine storm of sepsis results in a widespread apoptotic event, coupled with (and amplified by) the necrosis of damaged tissues and subsequent release of intracellular components (e.g. proteins and nucleic acids). This apoptotic event is mediated both cell-contact dependent (such as Fas-Fas ligand interactions) and cell contact-independent mechanisms (Mahidhara & Billiar, 2000). In regards to cell contact-independent mechanisms, instead of a single causative event, the combined stress of the multiple chemokines and cytokines upregulated by severe sepsis combine to induce apoptosis in multiple immune cell types. In addition, adverse coagulation events caused by unchecked activation of the complement cascade can also induce immune cell death, especially in the vasculature and susceptible immune organs (such as the bone marrow and spleen) (P. A. Ward, 2008). Within a relatively short time following the onset of sepsis – hours to days – primary and secondary lymphoid organs are significantly depleted of cells as a result of this apoptosis. For example, cell density is significantly reduced in the bone marrow, thymus and spleen during acute sepsis; this is especially striking in the thymus, where the organ shrinks in size dramatically during the early inflammatory response of severe sepsis (Riedemann et al., 2002; Wang et al., 1994). In addition, peripheral tissue-resident immune cells, such as dendritic cells (DCs), decrease in number during the acute phase of sepsis, in part due to cytokine storm-induced cell death(Tinsley et al., 2003).

The widespread immune cell apoptosis significantly limits the number of cells available to combat a secondary infection. Complicating this response is the limited activation potential of immune cells that survive the initial apoptotic event. As mentioned previously, severe

sepsis is induced through widespread activation of pattern recognition receptors (such as TLRs) and/or indiscriminate activation of nominally antigen-specific pathways (such as the case with polyclonal activation of T cells with superantigen). As a result of this excessive stimulation, the surviving immune cells become refractory to further stimulation through these specific pathways – this results in blunted responses to microbes and microbial products, leading to impaired immunity to secondary infections. This phenomenon is particularly evident in phagocytic cells of the innate immune system, manifested as a reduction in proinflammatory responses to secondary exposure to microbial products.

In macrophages, for example, prolonged exposure to high doses of LPS, as can be seen during severe sepsis, can result in refractory responses to further TLR signalling (Biswas & Lopez-Collazo, 2009). This suppressed TLR-mediated response is evidenced by a reduction in proinflammatory chemokine and cytokine production (Fujihara et al., 2003), as well as modulations in costimulatory ligand expression (Newton et al., 2004). This response is particularly prevalent in sepsis cases with a predominantly Gram-negative etiology, and high-dose LPS exposure in experimental models either *in vivo* or *in vitro* is often used to mimic this suppressed response (Medvedev et al., 2006). The phenomenon of "LPS tolerance" is intimately linked with the level of LPS exposure, as relatively low amounts (approximately 1 ng/ml in *in vitro* experiments) of LPS pre-treatment can enhance rather than suppress subsequent proinflammatory responses (West & Koons, 2008). However, high doses of LPS can result in suppressed extracellular signal-regulated kinase activity during subsequent secondary LPS exposures, including mitogen-activated protein kinases (MAPKs) and c-Jun NH_2-terminal kinase (JNK), with subsequent decreases in NF-κB and AP-1 transcription factor activity (Adib-Conquy et al., 2000; Fan & Cook, 2004; West et al., 2007). This decrease in TLR4-mediated signal transduction can lead to decreased production of proinflammatory genes such as Tumor Necrosis Factor-alpha (TNFα), interleukin(IL)-1β and IL-12, among others (Munoz et al., 1991; Spolarics et al., 2003).

Prolonged exposure to microbial products (and LPS in particular) also has a deleterious effect on the proinflammatory responses of neutrophils, although this response is distinct from the phenotype of "LPS tolerized" macrophages. Neutrophils play a critical role in initiating the septic response, as the early activation of neutrophils can lead to organ and tissue damage through the overproduction of reactive oxygen species (ROS) and highly active proteases such as neutrophil elastase (Fujimi et al., 2002; Sabroe et al., 2005). Additionally, accumulation of neutrophils in the vasculature and peripheral organs can result in non-specific cell and tissue damage, further complicating sepsis-induced organ dysfunction (Brown et al., 2006). Paradoxically, however, decreased neutrophil function during acute phase sepsis (characterized by decreased chemotactic responses and impaired surface receptor expression) is correlated with decreased survival in human patients, suggesting that the suppression of the antimicrobial functions of neutrophils is not enough to protect against sepsis-induced mortality (Chishti et al., 2004; Muller Kobold et al., 2000). Importantly, neutrophils are critical for the clearance of many nosocomial infections, as well as central to the innate immune responses against many experimental models of "two-hit" post-septic immunosuppression. Exhaustion of neutrophil functions during the acute phase of sepsis can therefore lead to susceptibility to secondary infections. For example, neutrophils that survive the early apoptotic event exhibit decreased chemotactic responses to neutrophil chemoattractants, most notably via CXCR1, which mediates neutrophil responses to CXCL8/IL-8 (Duffy et al., 2000). This reduced sensitivity to CXCL8 is similar to reduced TLR4 signalling in septic macrophages, as surface levels of CXCR1 are not reduced

on septic neutrophils; rather, the ability of CXCR1 to signal through G-protein coupled receptor (GPCR) signalling pathways appears impaired (Arraes et al., 2006; Reddy et al., 2008). Therefore, in a similar fashion to "LPS tolerized" macrophages, septic neutrophils are impaired in their ability to respond to secondary microbial stimuli, leaving the septic patient susceptible to secondary infections.

In addition, a form of passive immune suppression can be seen during early phase sepsis in the increase in immature neutrophils – also known as "band cells" or "banded neutrophils" – in peripheral blood and immune organs of patients and animal models. Banded neutrophils are immature neutrophils that have differentiated from granulocyte/monocyte precursors in the bone marrow, but have yet to fully differentiate into mature neutrophils (Klut et al., 1998). Increases in peripheral banded neutrophils have long been used as a simple diagnostic tool for inflammatory responses; while their use as a predictive tool for sepsis severity (and subsequent mortality) is under question, their presence as a result of severe inflammatory responses is well characterized (Cavallazzi et al., 2010; Cornbleet, 2002; M. J. Ward et al., 2010). The increase in peripheral banded neutrophils appears to be a response of the bone marrow to the widespread apoptosis of mature neutrophils in response to the cytokine storm of sepsis – however, while the newly released neutrophils are rapidly produced, they lack many of the functional aspects of fully differentiated neutrophils (Pillay et al., 2010). The increased number of banded neutrophils therefore may arise at the loss of fully differentiated and functional neutrophils. Just as the loss of leukocytes due to apoptosis renders septic patients and animal models susceptible to secondary infections, the increase of banded neutrophils can contribute to post-septic immunosuppression, as these cells are not able to control secondary infections in the same manner as mature, functional neutrophils.

It may appear paradoxical to identify cellular immune suppression in the context of uncontrolled inflammation, particularly in the population of peripheral innate immune cells (such as macrophages and neutrophils) that are largely responsible for the cytokine storm of severe sepsis. It is therefore important to consider that the peripheral innate immune compartment during the acute phase of sepsis is a heterogeneous mixture of pro-inflammatory cells, "exhausted" cells (as mentioned previously) and in addition, cells which have converted to a regulatory or suppressive phenotype in an attempt to counteract the uncontrolled inflammatory response. In the hours and days proximal to the onset of severe sepsis, this combination of immune cell phenotypes results in the multifaceted disease phenotype of sepsis, whereby uncontrolled inflammation both results in tissue damage and mortality, and renders bystander immune cells unable to respond to secondary infections.

3. Immune cell dysfunction during CARS and beyond

Despite the immunosuppressive phenomena discussed previously (LPS tolerance, chemokine desensitization, etc), the acute phase of sepsis is primarily defined by the over activation of proinflammatory mechanisms, in particular cytokine and chemokine production by activated immune cells. The widespread immune cell apoptosis and anergy (unresponsiveness to immunogenic stimuli) often fails to completely control the cytokine storm of sepsis; as a compensatory mechanism, the immune system attempts to switch to active mechanisms of immune suppression, in an attempt to resolve the SIRS response. These active mechanisms, including the production of anti-inflammatory cytokines and the generation, activation and differentiation of suppressor cells, are considered hallmarks of

the compensatory anti-inflammatory response syndrome (CARS) (A. Oberholzer et al., 2001). Classically, the CARS response was considered to be the generalized switch from pro- to anti-inflammatory cytokines during the acute phase of sepsis; this is evidenced by increased production of suppressive cytokines (such as IL-10) and cytokines which counteract the primarily T_H1-type inflammation of severe sepsis (such as T_H2 type cytokines IL-4, -5 and -13) (Miller et al., 2007). Recent epidemiological studies in human patients, along with studies of experimental animal models of sepsis, have identified the CARS response as being maintained in immune cells for extended timepoints, long after the resolution of sepsis. These responses still retain components of early-phase CARS, such as the T_H2 cytokine shift, but are also characterized by cell-intrinsic defects in activation, differentiation and effector function that blunt the effectiveness of post-septic immune cells to protect the host from opportunistic infections. In this conception, CARS can be defined as a multifaceted immunosuppression following the onset of severe sepsis that leaves the patient susceptible to increased morbidity and mortality as compared to healthy individuals. In a generalized categorization, early-phase CARS can be described in terms of directed immune cell suppression via cytokine and the directed activity of suppressor cells, while late-phase CARS can be described as cell-intrinsic defects in activation, differentiation and effector function.

3.1 Early phase CARS: Suppressor cells and immunosuppressive cytokines

The switch from SIRS to CARS represents a directed effort by the immune system to regulate the production of proinflammatory chemokines and cytokines that are mediating physiological stress and tissue damage. When considering the cellular mediators of SIRS, such as innate immune cells (e.g. macrophages and neutrophils), cell-extrinsic suppression of their effector function can proceed through cell contact-dependent and –independent mechanisms. In terms of cell contact-dependent mechanisms, the development and expansion of myeloid and lymphoid suppressor cells is one of the hallmarks of CARS, and the persistent presence of these cells can restrict cellular immune responses to secondary infections. In terms of cell contact-independent mechanisms, the production of suppressive cytokines (e.g. IL-10), cytokine-sequestering factors (e.g. soluble TNF receptor type II) and immune-deviating cytokines (e.g. IL-4 and IL-13) all serve to restrict immune responses to secondary infections as well. One important hallmark of these early phase responses is their direct correlation with the uncontrolled cytokine storm of SIRS; these early phase CARS responses are directly triggered by the proinflammatory cascade of acute sepsis, and may represent a final effort by the immune system to restrict the cytokine- and chemokine-driven inflammation of SIRS.

The development and expansion of suppressor cells during CARS is multifaceted, and encompasses both myeloid-derived and lymphoid-derived suppressor cells. In certain cases, the expansion of suppressor cells is due to the directed development of cells with suppressive phenotypes from bone marrow progenitor cells; this is postulated to be the case with the heterogeneous population of myeloid-derived suppressor cells (MDSCs) seen in the periphery of survivors of severe sepsis (Cuenca et al., 2011). In other cases, the relative expansion of cells with suppressive phenotypes is thought to be due to the deletion of effector cells during SIRS due to cytokine driven apoptosis; this is often described with the increase in peripheral regulatory T cells (T_{regs}) following sepsis (Venet et al., 2004). In either case, the resulting suppressor cells are defined by their ability to directly regulate the

activation of immune cells, including suppression of antigen presentation by accessory myeloid cells such as DCs, and restriction of the antigen-specific proliferation and effector function of T lymphocytes. The development of suppressor cells is a compensatory mechanism by the host to restrict the widespread inflammation of SIRS, both through biasing the peripheral immune cell pool towards cells with suppressive phenotypes, as well as utilizing those suppressive cells to directly regulate those proinflammatory cells that have failed to restrict their own activation through previously discussed mechansims (e.g. tolerance induction, apoptosis, etc).

Much of what is understood about MDSCs in humans comes from studies in cancer patients, where studies of peripheral blood leukocytes have identified a heterogeneous population of cells with stem cell markers on their surface (i.e. CD34) (Marigo et al., 2008). These cells are heterogeneous, both in terms of surface marker expression and in morphology, consisting of both monocyte and granulocyte subtypes. As a heterogeneous population, MDSCs have the ability to suppress the immune responses of other leukocytes, most notably T cells; co-cultures of MDSCs with CD4+ T cells result in decreased cell surface marker expression and cytokine production in response to antigen stimulation (Lechner et al., 2010). Their presence in the context of cancer is thought to arise through the modulatory activity of the malignancy, in an attempt to shield the growing tumor from immune surveillance (in a similar fashion as the development of tumor-associated macrophages) (Mantovani et al., 2009). In animal models of sepsis, MDSCs appear in peripheral immune organs in the days following experimental onset of the disease; these cells mimic the human MDSCs in their heterogenicity, their mixed monocyte and granulocyte morphology, and their ability to suppress antigen-specific lymphocyte responses *in vitro* (Delano et al., 2007).

While there remains a paucity of data concerning direct observation of MDSCs in human patients during severe sepsis and following recovery, techniques have been developed to generate MDSCs from peripheral blood mononuclear cells, utilizing a cocktail of cytokines that are also key players in the cytokine storm of severe sepsis (such as IL-6, IL-1β and TNF-α) (Lechner et al., 2010). The majority of experimental data concerning MDSCs in sepsis comes from experimental models, especially in the mouse – peripheral MDSCs can be induced by high dose LPS treatment, and these cells exhibit multifaceted immunosuppressive capabilities, including suppression of antigen-specific responses (Bronte, 2009; De Wilde et al., 2009). The source of peripheral MDSCs appears to be from progenitor cells in the bone marrow, although their presence as a terminally differentiated cell population vs. a mobilized population of immature leukocytes in response to inflammation and subsequent peripheral cell apoptosis (often referred to as "emergency myelopoiesis") remains in question. In addition, their role as mediators of post-septic immunosuppression is unclear, both due to the paucity of MDSC data from human septic patients, and in the difficulty inherent in eliminating MDSCs from the periphery of experimental animals. This difficulty arises from the lack of a definitive marker for MDSCs, in particular one that is not shared by other immune cells (e.g. macrophages and neutrophils). For example, many published studies in animal models utilize anti-Gr1 antibodies to deplete peripheral MDSCs to study their impact on disease (Nausch et al., 2008; Ribechini et al., 2009); however, this antibody also depletes mature neutrophils, and anti-Gr1 treatment on its own can render mice susceptible to opportunistic infections due to the depletion of these neutrophils (Mehrad et al., 1999). However, the correlative data from human patients, in context with the studies in animal models of sepsis, suggest that severe

sepsis can result in an increase in peripheral MDSCs. When coupled with their ability to suppress antigen-specific activation and effector function of lymphocytes, MDSCs become a strong candidate for mediating post-septic immunosuppression.

In addition to myeloid-based suppressive cells, restriction of immune cell function post-sepsis is mediated by suppressive lymphoid cells as well. Arguably the best understood of these suppressive lymphocytes is the CD4+ regulatory T cell (T_{reg}). T_{regs} are themselves relatively heterogeneous, based on cell surface marker phenotype, tissue of residence, and/or the disease (or disease model) where the cells are studied. While T_{regs} are nominally defined by their suppressive activity (both *in vivo* and *in vitro*), they are identified by the expression of the transcription factor Foxp3 (Campbell & Ziegler, 2007), and are correlated with the expression of specific cell surface markers (most notably CD25, but also CTLA-4 and GITR, among others) (Wilczynski et al., 2008). T_{regs} have the capacity to suppress antigen-specific responses by effector T cells, as well as inflammatory responses by innate immune cells (Suzuki et al., 2010). T_{regs} are critical for the maintenance of peripheral immune tolerance, and deletion of T_{regs} in animal models results in the development of autoimmunity (Suri-Payer & Fritzsching, 2006). In humans, genetic mutations in the *Foxp3* gene results in the development of autoimmune disease (i.e. IPEX syndrome), due to a breakdown of T_{reg}-mediated suppression of self-antigen responses in the periphery (van der Vliet & Nieuwenhuis, 2007). Additionally, accumulations of T_{regs} in peripheral organs can help suppress immune responses and promote tolerance induction; examples of this phenomenon include inflammatory bowel disorders (Uhlig et al., 2006), allergic asthma (W. F. Carson et al., 2008) and transplant tolerance (Ge et al., 2010). In a negative context, peripheral T_{regs} can also suppress tumor surveillance by the adaptive immune system, indicating that in certain disease contexts, T_{regs} can promote rather than reduce pathology (Nishikawa & Sakaguchi, 2010).

Phenotypic analysis of peripheral blood from patients suffering from severe sepsis indicates an increase in CD4+ T_{regs} (Venet et al., 2009). This increase is seen rapidly following the onset of disease, and can persist throughout recovery (defined as the resolution of the cytokine storm), especially in animal models of sepsis. The origin of the increased T_{regs} is controversial, as certain studies describe the increase as being a directed expansion of T_{regs} and others as a result of the depletion of effector T cells due to sepsis-induced apoptosis (Venet et al., 2004; Wisnoski et al., 2007). Interestingly, *in vivo* studies utilizing PBMCs indicate that bacterial SAgs have the capacity to induce Foxp3 expression, suggesting that certain types of polyclonal stimuli associated with severe sepsis can directly modulate T_{reg} numbers (Taylor & Llewelyn, 2010). Regardless of the nature of this increase, however, it is clear that peripheral T_{regs} are enhanced in number in post-septic patients. At present, experimental modulation in T_{regs} has focused on their role in mediating the severity of acute sepsis; however, recent studies have begun to shed light on the role of T_{regs} in mediating post-septic immunosuppression. For example, increases in peripheral T_{reg} numbers in humans correlates with the development of suppressive phenotypes in peripheral lymphocytes from the same patients (Monneret et al., 2003). In animal models of sepsis, *ex vivo* blockade of Foxp3 via treatment with silencing RNAs (siRNA) can restore the proliferative responses of splenocytes, suggesting that Foxp3-dependent mechanisms are mediating lymphocyte anergy in post-septic animals (Venet et al., 2009). The direct effect of peripheral T_{regs} in mediating post-septic immunosuppression remains unclear; however, similar experimental models of secondary infections following trauma suggest an important role for T_{regs} in mediating immunosuppression following uncontrolled inflammation. For

example, in animal models of severe burn injury, secondary infection with *Pseudomonas aeruginosa* results in increased mortality as compared to healthy control mice. Importantly, experimental neutralization of T_{reg} *in vivo* in these animals reverses this susceptibility (Liu et al., 2011).

Of particular interest to the study of post-septic immunosuppression is the nature of T_{reg} generation. Functional T_{regs} can be generated in two distinct pathways; either in the thymus ("natural" T_{regs}) or in the periphery in response to antigen stimulation in the context of tolerance-promoting signals, such as immunosuppressive cytokines ("adaptive" or "induced" T_{regs}) (Bluestone & Abbas, 2003). As mentioned previously, one possible explanation for the observed increase in peripheral T_{regs} following sepsis is the concurrent apoptosis of effector T cells; this would suggest that it is natural T_{regs} that are the primary regulatory T cell type following sepsis. However, studies of peripheral CD4+ T cells in animal models of sepsis suggest that following the onset of inflammation, peripheral effector T cells may have an increased propensity to become T_{regs} in response to secondary stimulus, due to modulations in their gene regulation patterns (Cavassani et al., 2010). This suggests that severe sepsis may condition the peripheral T cell pool to respond in a more regulatory fashion to secondary antigen challenges, which would ultimately result in an increase in immune suppression at the cost of protective pro-inflammatory responses. These modulated gene regulatory events will be discussed in the following sections dealing with late-phase CARS, as similar gene regulatory events underlie cell-intrinsic defects in the activation and effector function of other leukocytes during long-term sepsis-induced immunosuppression.

In concert with the increase in suppressive immune cells, imbalances in cytokine production by the immune system during the switch from SIRS to CARS can also contribute to post-septic immunosuppression. Characterization of the upregulation of these soluble mediators comes from studies at the physiological level, i.e. analysis of peripheral blood samples from human patients or peripheral tissues from animal models. The relative increase in these soluble anti-inflammatory mediators is observed in survivors of severe sepsis, and the presence of these mediators has a profound effect on the ability of leukocytes to respond to secondary infections. Soluble mediators of post-septic immunosuppression can be grouped into three general categories based on their mechanism of action: anti-inflammatory cytokines, soluble cytokine-sequestering receptors and immune-deviating cytokines. While the ultimate function of each grouping is different (i.e. direct suppression vs. immune-deviating), all three types of soluble factors contribute to post-septic immunosuppression.

The most well understood of the many immunosuppressive cytokines produced during CARS is arguably IL-10 (Bazzoni et al., 2010). Signalling via IL-10 proceeds through a heterodimeric receptor (IL-10R1/IL-10R2 complex), and the intracellular signalling pathways associated with IL-10 receptor activation (including involvement of tyrosine kinases and the transcription factor STAT3) have the ability to directly regulate cytokine gene expression induced by LPS; this makes IL-10 an intriguing candidate for mediating post-septic immunosuppression, especially in regards to LPS-dependent secondary responses (Crepaldi et al., 2001). Supporting this concept is the kinetics of IL-10 expression in human patients and animal models, where increased levels of IL-10 can be observed following the onset of sepsis (van der Poll et al., 1997). Blockade of IL-10 has been shown to have a protective effect in many experimental models of severe sepsis (Latifi et al., 2002; Oberholzer et al., 2002). Interestingly, however, increased IL-10 production in human patients is often correlated with poor outcomes, suggesting that IL-10 may be a potent

regulator of the CARS response (Abe et al., 2008). IL-10 has potent suppressive effects on a wide range of leukocytes, including suppression of proliferation, surface receptor expression and cytokine/chemokine production (Akdis & Blaser, 2001; Williams et al., 2004). Of particular interest is IL-10's potent effect on monocytes and macrophages, primarily mediated by the antagonistic effect of IL-10 signalling on LPS-dependent gene induction (Cavaillon & Adib-Conquy, 2006). IL-10 has dramatic suppressive effects on the pro-inflammatory responses of macrophages, including inhibition of cytokine production (Brandtzaeg et al., 1996; Gerard et al., 1993). IL-10 can also negatively impact neutrophil activation in the context of LPS stimulation (as seen with Gram-negative sepsis), as neutrophils only expresses the IL-10R following LPS stimulation; in this fashion, IL-10 serves as a negative regulator of activated neutrophils (Cassatella et al., 2005; Tamassia et al., 2008). In a normal physiological context, limited by either the kinetics of stimulation or the restricted microenvironment of a given inflammatory insult, the IL-10-dependent negative feedback look allows the immune system to properly regulate the potentially damaging anti-inflammatory effects of macrophages and neutrophils following the clearance of infectious micro-organisms. However, the high levels of IL-10 produced during CARS can drastically shift the balance away from pro-inflammatory responses, leaving peripheral macrophages and neutrophils unable to respond to secondary bacterial challenges unrelated to the causative agent of septic shock.

In addition to IL-10, the shift from SIRS to CARS is also associated with an increase in transforming growth factor-beta (TGFβ). TGFβ is a potent cytokine that is normally involved with wound healing and the development of fibrosis in damaged tissues (Leask & Abraham, 2004). TGFβ also has the ability to direct the activation and differentiation of many cell types, including but not limited to immune cells. In the context of immune responses, TGFβ is a potent immunosuppressive cytokine, with the ability to suppress the antigen-specific activation and effector function of leukocytes (Prud'homme & Piccirillo, 2000). Additionally, TGFβ signalling in the context of T-cell receptor signalling can direct the development of CD4+ T_{regs} from effector T cell precursors (Li & Flavell, 2008). In the context of severe sepsis, TGFβ is significantly increased in the systemic circulation (Marie et al., 1996); this increase is both due to the directed production of TGFβ by immune cells, but also as a byproduct of sepsis-induced apoptosis, as TGFβ is produced by apoptotic cells (T cells in particular) during programmed cell death (Chen et al., 2001). TGFβ isoforms (of which there are 3 primary forms) signal through cell surface receptors through SMAD transcription factors, which regulate numerous genes, including those responsible for mediating apoptosis (for example, through downregulation of anti-apoptotic genes such as Bcl-xl) (Kanamaru et al., 2002; Spender et al., 2009). TGFβ signalling can also limit chemokine and cytokine production by immune cells, in particular T lymphocytes and activated macrophages (Letterio, 2005; Yang et al., 2010). In particular, TGFβ is a potent suppressor of the anti-microbial activity of macrophages; this is manifest via TGFβ-mediated suppression of cytotoxic activity, superoxide production and nitric oxide synthase (iNOS) activity (Mitra & Khar, 2004). This increase in TGFβ during sepsis can therefore suppress secondary anti-microbial responses by macrophages, limiting the ability of these cells to respond to secondary infections. In addition, as TGFβ signalling is important for the differentiation of effector CD4+ T cells into T_{regs}, there appears to be a role for increased TGFβ production post-sepsis in mediating the increase in peripheral T_{regs} observed in human patients and animal models. Therefore, in addition to TGFβ's direct immunosuppressive properties (as with IL-10), TGFβ can also mediate immunosuppression through the development of cells with intrinsic immunosuppressive properties.

The immunosuppression observed during early phase CARS proceeds both through the production of anti-inflammatory cytokines, but also through the production of soluble factors that can limit the ability of pro-inflammatory cytokines to signal cells and mediate inflammation. Of particular importance to the switch from SIRS to CARS is the production of soluble receptors and antagonists that interfere with the signalling of the proinflammatory cytokines IL-1β, IL-6 and TNFα. All three cytokines are central mediators of SIRS through their ability to drive the acute phase response, fever, cytotoxic activity of immune cells (e.g. phagocytosis) and ultimately septic shock (Jean-Baptiste, 2007). As a result, during the switch from SIRS to CARS, the immune system upregulates the production of cytokine antagonists which attempt to block the activity of these inflammatory mediators. For example, in septic patients, plasma levels of soluble IL-1ra receptor antagonist (IL-1ra), soluble TNFα receptors I and II (sTNFRI/sTNFRII), and soluble IL-6 receptor (sIL-6R) are all increased as compared to healthy patients (Gogos et al., 2000; Marie et al., 1997). IL-1ra binds the IL-1 receptor in competition with IL-1β, resulting in functional inhibition of IL-1β signalling (Bresnihan & Cunnane, 1998). In a similar fashion, the sTNFRs and sIL-6R achieve functional inhibition of their respective cytokines by binding and sequestering the soluble proteins from interacting with cell surface receptors on circulating leukocytes (Jones & Rose-John, 2002). As with the upregulation of anti-inflammatory cytokines, the production of receptor antagonists and soluble receptors is an attempt by the immune system to restrict the cytokine storm of SIRS. However, blockade of the functional activity of these cytokines can result in vulnerability to secondary infections, especially when placed in context with the upregulation of anti-inflammatory cytokines such as IL-10 and TGFβ.

For cellular immune responses to be protective, they must be conditioned to produce the proper inflammatory response for the specific infectious agent. The cytokine storm of SIRS can be loosely categorized as a T helper type-1 response (T_H1), geared towards cytotoxic activity and anti-microbial responses. Many cytokines central to sepsis responses, such as TNFα, IL-6, IL-12 and IFNγ, are normally considered to be T_H1-type cytokines, as they promote the cytotoxic activity of macrophages, neutrophils and CD8+ cytotoxic T cells. T-helper type-2 responses (T_H2) are associated with immunity to parasites, as well as allergic responses, and are characterized by antibody production (primarily immunoglobulin type E) and eosinophilopoiesis (at the expense of neutrophils). T_H2 cytokines have the ability to directly regulate T_H1 responses through their ability to suppress T_H1 gene expression in CD4+ T cells, as well as by modulating the granulocyte output of the bone marrow in response to inflammation (i.e. eosinophils vs. neutrophils) Summaries of the T-helper cytokine response and its role in inflammation are available in the literature (Cameron et al., 2001; DiPiro, 1997; Miller et al., 2007). As a third cytokine-dependent mechanism, the cytokine milleu of CARS is also characterized by an upregulation of T_H2 cytokine responses (Mack et al., 1996). This upregulation of T_H2 cytokines serves to blunt the T_H1-dominant SIRS response through the modulation of leukocyte gene expression. In human patients, immune responses post-sepsis are observed to have a generalized shift towards T_H2 responses, especially in regards to adaptive immune cell activation (O'Sullivan et al., 1995). IL-4 in particular has potent effects on macrophages, with the ability to suppress cytotoxic activity as well as promote the production of IL-1ra and sTNFRs, resulting in an autocrine feedback loop that further restricts macrophage activity (Nicod et al., 1994). In addition, T_H2 cytokines like IL-4 and IL-13 are key factors in the development of alternatively activated macrophages (aaMΦ, or M2-type macrophages), which promote wound healing and fibrosis

at the expense of anti-microbial activity (Gordon & Martinez, 2010). Animal models of sepsis have identified that bone-marrow derived macrophages from survivors of sepsis exhibit a M2 phenotype as compared to control animals, suggesting that the T_H2 cytokine-dependent development of M2 macrophages can be maintained for timepoints distal from the acute inflammatory event (Takahashi et al., 2004).

Because a majority of the studies dealing with soluble mediators of the CARS phenotype are done on the physiological level (i.e. with analysis of peripheral blood samples), it is often difficult to directly identify the source of the immunomodulatory cytokines mediating CARS. In the case of soluble receptors and receptor antagonists, the root source is often considered to be macrophages and other phagocytotic innate immune cells, especially in response to circulating IL-10 levels. As mentioned previously, increases in TGFβ production are often the result of lymphocyte apoptosis during sepsis; however, many T cells, especially T_{regs}, can produce both TGFβ and IL-10 when mediating immunosuppression. Many of the T_H2 cytokines observed during CARS are normally produced by CD4+ T cells, and it is hypothesized that these cells are at the root of the T_H12 shift observed in survivors of severe sepsis. Conceptually, the shift from SIRS to CARS may result from a negative feedback loop whereby the proinflammatory cytokine storm results in the apoptosis of cells, resulting in increased TGFβ production that then leads to the development of T_{regs} that produce IL-10, which then go on to stimulate anti-inflammatory responses (i.e. IL-1ra, sTNFR & sIL-6R) from macrophages, and so on. This type of model may explain the mechanisms underlying the switch from SIRS to CARS; however, they do not fully explain the maintenance of post-septic immunosuppression in patients for the months and years following recovery. Investigations into the long-term maintenance of the CARS phenotype have identified many cell-intrinsic defects in activation and effector function that maintain the immunsuppressive phenotype once the relative levels of anti-inflammatory cytokines and antagonistic factors have returned to baseline levels in the periphery. It is hypothesized that these cell-intrinsic factors may be programmed by the cytokine milleu of either SIRS or CARS; additionally, they may utilize modulation in gene regulation as an underlying mechanism for the maintenance of these defects.

3.2 Late phase CARS: Cellular dysfunction and epigenetic reprogramming

Sepsis-induced immunosuppression during SIRS and the early switch to CARS relies on active mechanisms, such as cytokine-induced apoptosis, anergy induction, suppression of signal transduction and modulation of bone marrow hematopoiesis. Ultimately, however, these mechanisms of suppression resolve as the patient recovers from the septic episode. For example, despite the widespread apoptosis & necrosis of immune cells observed during the acute phase of sepsis, immune cells are ultimately re-seeded into immune tissues and peripheral blood & lymph following recovery. Interestingly, the re-seeding of T lymphocytes to peripheral organs does not appear to favor specific T cell-receptor subtypes (Unsinger, Kazama, et al., 2009). This phenomenon suggests that the repopulation of lymphocytes is not due simply to the expansion of cells primed during the acute phase of septic shock. However, despite the return of the immune system to a state comparable to conditions pre-sepsis, both human patients and experimental animals continue to exhibit signs of immunosuppression. As mentioned previously, the long term survival curves of survivors of severe sepsis are significantly reduced as compared to the healthy age-matched population, and this increased morbidity and mortality is observable even in the presence of

co-morbidities. Additionally, self-reported quality of life measurements are significantly reduced in survivors of severe sepsis, indicating negative physiological outcomes even in the absence of secondary infection. Taken in context, these results suggest that the cellular immune response in post-septic individuals remains dysregulated even once the major mediators of CARS have subsided.

Experimental studies aimed at identifying cell-intrinsic defects in activation and effector function of immune cells following severe sepsis have described numerous immunosuppressive phenomena that are maintained even after the resolution of SIRS and CARS. These deficiencies in activation and effector function are considered to be cell-intrinsic as they manifest both *in vivo* and *in vitro*, are often preserved in adoptive transfer models, and are in many cases resistant to treatment with optimized culture conditions (e.g. polyclonal stimulus and exogenous recombinant cytokines). In "two-hit" models of post-septic immunosuppression, these cell-intrinsic defects contribute directly to the susceptibility of survivors of severe sepsis to opportunistic infections, as adoptive transfer of immune cells from control animals can confer protect in these immunosuppressed animals. The current working hypothesis for this phenomenon deals with gene regulation in post-septic cells, in particular with the expression of proinflammatory cytokines that are necessary for protection against secondary infection. In this model, immune cells that have survived septic shock are no longer able to effectively respond to secondary infectious stimuli due to the repression of gene expression following activation. While many disparate yet interrelated molecular mechanisms may be involved in this dysregulation of gene expression, recent studies have implicated epigenetic reprogramming of immune cells (and possibly hematopoietic progenitor cells) as a major player in long-term immunosuppression post-sepsis (W. F. Carson et al., 2011).

Perhaps the most well-characterized immune cell deficiency following sepsis is concerning the activation of DCs. As mentioned previously, DCs are rapidly depleted from peripheral tissues following the onset of sepsis, and as DCs are critical for the antigen-specific activation of T cells, this loss of peripheral DCs severely restricts the activation potential of the adaptive immune system (Tinsley et al., 2003). Interestingly, peripheral DCs that do return following the resolution of SIRS and early-phase CARS continue to exhibit an immunosuppressive phenotype following sepsis, highlighted by a significant reduction in their ability to produce IL-12 in response to TLR stimulation (Wen et al., 2006). IL-12 is a potent pro-inflammatory cytokine that is critical for the development of T_H1 responses and T_H1-type CD4+ T cells (Watford et al., 2003). This inability to produce IL-12 results in susceptibility to pathogens that are normally cleared by a T_H1 immune response. Experimentally, this response can be studied using airway challenges with *Aspergillus fumigatus* in post-septic mice – the reduction in DC-dependent IL-12 production in these animals restricts the development of T_H1 responses, resulting in uncontrolled fungal growth and *Aspergillus*-induced mortality (Benjamim et al., 2003). When bone-marrow derived DCs from control animals are transferred into post-septic animals prior to airway challenge, resistance to fungal infection can be restored (Benjamim et al., 2005). Interestingly, this suppression of IL-12 production does not appear to be due to increased IL-10 production; while *ex vivo* stimulated post-septic DCs exhibit increased IL-10 production (an expected CARS-type cytokine response), neutralization of IL-10 *in vitro* with blocking antibodies fails to restore IL-12 production (Wen, Dou, et al., 2008). Importantly, this suppression of IL-12 production is maintained long past the resolution of severe sepsis, with lung DCs exhibiting

deficiencies in IL-12 production up to six weeks following the experimental onset of sepsis (Wen, Dou, et al., 2008). From a therapeutic standpoint, this long-term suppression of IL-12 production is problematic, as it does not follow from the upregulation of anti-inflammatory cytokines that can themselves be blocked (as with *in vitro* inhibition of IL-10).

DCs are critical accessory cells for the activation of T cells, through the presentation of antigen to T-cell receptors in the context of MHC. In addition, DCs instruct T cells to differentiate into one of several effector/regulatory cell lineages (characterized by distinct families of effector cytokines and downstream immunomodulatory functions); this instruction occurs both through cell-contact dependent mechanisms and the production of instructional cytokines (such as IL-12). As mentioned previously, post-septic immune responses are characterized by a generalized shift away from T_H1 cytokines towards T_H2 cytokines. This phenomenon may be due at least in part to the immunomodulatory properties of post-septic DCs, as these cells promote T_H2 cytokines such as IL-4, IL-5 and IL-13 at the expense of T_H1 cytokines such as IFNγ (Wen et al., 2006). This skewing to T_H2 occurs in an antigen-specific fashion, and is not a result of pre-conditioning of the responder T cells during severe sepsis, as the responder cells used in these *in vitro* experiments were naïve and specific for an antigen that is unrelated to polymicrobial sepsis (ovalbumin). This reduction in T_H1 polarizing capabilities may also negatively regulate innate immune system functions, as T_H1 CD4+ T cell-derived IFNγ is critical for the activation of phagocytotic activity by macrophages (Kasten et al., 2010).

In addition to DC-mediated suppression, CD4+ T cell exhibit deficiencies in activation, differentiation and effector function following sepsis, even in the context of polyclonal stimulus or nominally functioning DCs. One of the hallmarks of CD4+ T cells from septic patients is the development of anergy, or unresponsives to antigen stimulation (Heidecke et al., 1999). CD4+ T cells from septic animals also exhibit this anergic phenotype, and the proliferative capacity of these cells is not recovered by the addition of exogenous cytokines, such as the potent T cell proliferative factor IL-2 (W. F. Carson et al., 2010). In addition, their gene expression is drastically altered as compared to CD4+ T cells from healthy animals. For example, mRNA for IFNγ, IL-4 and the T_H17 cytokine IL-17 can be upregulated in naïve CD4+ T cells from post-septic mice following polyclonal stimulus, as compared to levels in cells from control animals. Concurrently, these cells downregulate mRNA for cell surface receptors critical for T cell activation, including CD4 and CD28. As these differences in gene expression are observed following optimized polyclonal stimulus in the absence of accessory cells such as DCs, it appears that the changes in gene expression are due to cell-intrinsic factors (W. F. Carson et al., 2010).

This modulated cytokine response by post-septic CD4+ T cells becomes even more apparent once these cells are tasked with committing to specific T-helper lineages. When studied *in vitro*, post-septic CD4+ T cells exhibit deficiencies in their ability to properly commit to either the T_H1 or T_H2 lineage, as evidenced by cytokine production. For example, when expanded in an optimized T_H1-promoting environment (including exogenous IL-12 and blocking antibodies to IL-4), post-septic CD4+ T cells produce significantly less IFNγ in recall responses, as compared to CD4+ T cells from control animals. If these same cells are expanded in a T_H2 culture (exogenous IL-4 and blocking antibodies to IL-12 and IFNγ), post-septic CD4+ T cells produce both T_H1 and T_H2 cytokines upon recall, whereas CD4+ T cells from control animals only make T_H2 cytokines (W. F. Carson et al., 2010). This modulation in T-helper subtype cytokine responses represents an inability of post-septic CD4+ T cells to properly commit to either the T_H1 or T_H2 effector lineage.

This modulated cytokine production is also apparent *in vivo*, when post-septic CD4+ T cells are transferred into lymphopenic animals (i.e. Rag2$^{-/-}$ mice, which lack mature peripheral lymphocytes). As mentioned previously, post-septic myeloid cells exhibit numerous deficiencies in activation and cytokine production throughout SIRS, early and late phase CARS; as these cells are critical for the activation of CD4+ T cells, any deficiencies observed in lymphocyte activation *in vivo* in post-septic animals may be due to myeloid deficiencies, and not necessarily due to CD4+ cell-intrinsic factors. Adoptive transfer experiments allow post-septic CD4+ T cells to be studied in the context of a functional myeloid immune cell response, as both macrophages and DCs develop and function normally in (genetically engineered) lymphopenic mice. When post-septic CD4+ T cells are challenged with *in vivo* models of T_H1 or T_H2 lung inflammation, a similar pattern of cytokine expression emerges. Histological examination of lung granuloma formation in response to embolized antigen-coupled beads indicates that post-septic CD4+ T cells mediate smaller T_H1 and T_H2 type lesions, as expected based on the generalized shift to T_H2 responses following sepsis (W. F. Carson et al., 2011). Interestingly, when lymph nodes from mice that received post-septic CD4+ T cells were restimulated with cognate antigen, they produced increased amounts of a wide range of T-helper cytokines, including IL-4 in a T_H1 context, and IFNγ in T_H2 context. Additionally, in both T_H1 and T_H2 disease models, lymph nodes from mice that received post-septic CD4+ T cells produced increased IL-17 as compared to those that received CD4+ T cells from control mice. In both cases, this inability to properly commit to a T-helper lineage results in measurable differences in both *in vivo* inflammatory processes and *ex vivo* cytokine production (W. F. Carson et al., 2011). While it may be counter-intuitive to suggest that increased pro-inflammatory cytokine production is indicative of immunosuppression, it is important to place this increase in the context of productive inflammation. Adaptive immunity requires directed cytokine production to initiate proper immune responses directed against the specific infectious agent (i.e. T_H1 responses for intracellular bacterial and viral infections vs. T_H2 responses for helminth infections). Increases in T-helper cytokines can be counterproductive when they promote incorrect inflammatory responses (as seen with the T_H1 granuloma studies) or exacerbate inflammation past the level of protection (as seen with the T_H2 granulomas). When coupled with the shift towards T_H2 responses observed with DCs post-sepsis, this dysregulation of cytokine expression can ultimately result in decreased immunity to secondary infections, which normally require T_H1 responses for clearance.

During late phase CARS, both myeloid cells (specifically DCs) and lymphoid cells (specifically CD4+ T cells) exhibit deficiencies in cytokine production in response to stimulation with microbes, microbial products and antigens. As mentioned previously, following the apoptotic response during SIRS, both DCs and CD4+ T cells are re-seeded to the periphery, in many cases to levels similar to those found in control animals or healthy individuals. These results suggest that the mechanisms governing cytokine responses in post-septic cells are both cell-intrinsic and not reliant on exogenous factors, such as soluble mediators of early phase CARS (e.g. IL-10, IL-1ra, etc). Recent studies in animal models have identified one possible molecular pathway governing this dysregulation of gene expression and cytokine production, epigenetics. The study of epigenetics involves any and all molecular mechanisms that can modulate gene expression without changing the underlying genetic information present in a cell or organism. Epigenetic mechanisms include chemical modifications to DNA and DNA-associated histone proteins that can either activate or suppress gene transcription, as well as expression of small RNA species (called

microRNAs/miRNAs) that can post-transcriptionally regulate gene expression through the targeted degradation of mRNA (Delcuve et al., 2009). Epigenetics plays a critical role during embryogenesis and development, particularly when multipotent stem cells commit to defined lineages during the development of tissues and organs (Roloff & Nuber, 2005; Shafa et al., 2010). The differentiation process requires tight regulation of gene expression, as the differentiation of stem cells into cells with defined phenotypes requires not only the activation of essential genes, but also the suppression of genes unrelated to the function of the new daughter cell. In a similar fashion, the immune system relies on epigenetic mechanisms to guide the development of peripheral blood leukocytes from hematopoietic cell precursors (Bergman & Cedar, 2010; Fernandez-Morera et al., 2010). In addition, the activation, differentiation and effector function of peripheral immune cells also relies on epigenetic mechanisms. For example, modifications in the tails of histones associated with the promoter regions of essential gene families correlates with the decision of macrophages to become either classically activated (i.e. anti-microbial) or alternatively activated (i.e. fibrotic/wound healing) (Ishii et al., 2009). In addition, CD4+ T cells utilize similar histone modification processes to govern the expression of T-helper cytokines during the differentiation to T_H1 or T_H2 (Ansel et al., 2003). When these processes are disrupted in immune cells, disease can often result; for example, modulations in DNA methylation patterns (DNA methylation results in the suppression of gene expression) are often correlated with the development of systemic lupus erythematosus (D. R. Patel & Richardson, 2010). Regulation of gene expression is essential for the proper function of the immune system, and changes in the epigenetic landscape of an immune cell can have a profound effect on immune responses and subsequent disease.

Recent studies in animal models have identified a number of epigenetic modifications in immune cells following severe sepsis. These modifications are often found associated with genes essential for immune cell effector function, such as cytokine genes or transcription factors, and they correlate with deficiencies in activation, differentiation and effector function in these cells. Additionally, these modifications are found in both myeloid and lymphoid cells, suggesting a common molecular mechanism for the modification of epigenetic marks in leukocytes following sepsis. At present, the best described epigenetic mechanism governing post-septic immunosuppression is via histone modification events, in particular changes in histone acetylation and methylation. Post-translational modifications of the protein tails of histone core proteins can have a direct effect on gene expression, depending on the type of modification and its location on the histone tail (Cosgrove & Wolberger, 2005). For example, acetylation of histone tails is often associated with transcriptional activation, regardless of the location of the modification on the histone tail. In contrast, the functional result of the methylation of histone tails is site specific; transcriptional activation or repression can occur depending on the location of the methylation event on the histone tail. Studies of both myeloid and lymphoid cells post-sepsis have identified modulations in both histone acetylation and methylation following sepsis, in particular in the promoter regions of genes essential for mediating protective immune responses against secondary infections.

In DCs, for example, modulations in histone methylation events in the promoter regions of IL-12 are observable following sepsis (Wen, Schaller, et al., 2008). When tissue-resident DCs from post-septic animals are compared to those from healthy controls, the pattern of histone methylation is skewed towards gene repression. This includes decreases in methylation of lysine 4 on the tail of histone 3 (H3K4me), which is considered an activating epigenetic

mark, as well as increases in methylation of lysine 27 on the tail of histone 3 (H3K27me), which is considered a repressive epigenetic mark. These marks are maintained for a significant amount of time following the resolution of severe sepsis, as tissue-resident DCs from post-septic animals retain these modulated methylation marks six weeks after the experimental onset of severe sepsis. In addition, the chromatin modifying enzymes responsible for the addition of these histone methylation marks is significantly altered in post-septic DCs, with the H3K4me machinery decreased and the H3K27me machinery increased in the promoter regions of both IL-12a and IL-12b . This increase in repressive histone methylation marks correlates with decreased IL-12 production both *in vitro* and *in vivo*, and provides one explanation for the observed reduction in proinflammatory cytokine production that is unrelated to the presence of anti-inflammatory cytokines (i.e. IL-10) (Wen, Dou, et al., 2008).

While the data concerning epigenetic reprogramming in macrophages is less robust in regards to long-term immunosuppression, modulations in histone modifications can be observed in macrophages following the onset of severe sepsis, and these modulations affect proinflammatory responses by these cells. For example, macrophages recovered from experimental models of severe sepsis exhibit increased levels of methylation at lysine 9 of histone 3 (H3K9, repressive) at the promoter region of both IL-1β and TNFα, resulting in decreased production of both inflammatory mediators (Lyn-Kew et al., 2010). This increase in repressive histone methylation appears to be a mechanism whereby the immune system is attempting to limit proinflammatory cytokine production; however, as both of these cytokines are critical for immunity against bacteria, their decreased production by macrophages can leave the immune system less able to properly respond to secondary infections. In addition, decreases in H3K4me and increases in acetylation of the tail of histone 4 (AcH4) are observed both in the TNFα and iNOS promoter; reductions in iNOS expression severely limit the ability of the macrophage to produce nitric oxide, a critical component of the cytotoxic response. To date, few studies have been performed to determine if these aberrant histone modification events remain present in macrophages long after the resolution of SIRS. However, recent studies identifying an epigenetic role for the development of the alternatively activated macrophage (decreases in H3K27 methylation mediated by the histone demethylase KDM6B) may provide a mechanism for the increased propensity of post-septic macrophages to become alternatively activated, as the increase in KDM6B expression and activation is driven by T_H2 cytokines that are upregulated during CARS, most notably IL-4 (Ishii et al., 2009).

Epigenetic reprogramming of CD4+ T cells following sepsis is also apparent, both in regards to cytokine production and T-helper lineage commitment. As mentioned previously, CD4+ T cells from post-septic animals exhibit deficiencies in their ability to properly commit to either the T_H1 or T_H2 lineage, as evidenced by modulations in cytokine production during recall/re-challenge both *in vivo* and *in vitro*. These deficiencies in lineage commitment correlate with modulations in histone modifications, specifically increases in H3K27me levels in promoter regions of T-helper subtype genes. For example, post-septic CD4+ T cells exhibit increased levels of H3K27me in the promoter region of *Ifng*, correlating with the decreased production of IFNγ during skewing and restimulation experiments *in vitro*. In addition, these same cells exhibit increased levels of H3K27me in the promoter region of *Gata3*, a transcription factor critical for the development of T_H2 T cells. This increase in repressive histone methylation correlates with the reduced capacity of post-septic T cells to fully commit to the T_H2 lineage, as evidenced by the continued production of IFNγ in T_H2

inflammatory contexts both *in vitro* and *in vivo (W. F. Carson et al., 2010; W. F. Carson et al., 2011)*. In addition to modulations in histone methylation, histone acetylation is also modulated in post-septic CD4+ T cells, in particular the CD4+ CD25- T cell subset that is thought to consist primarily of effector cells. CD4+ CD25- T cells from post-septic animals exhibit increases in H3K9 acetylation (H3K9ac) in the promoter region of *Foxp3* (Cavassani et al., 2010); Foxp3 is a transcription factor considered to be a master regulator of T_{reg} function, and expression of Foxp3 is essential for the suppressive activity of T_{regs}. In addition, CD4+ CD25- T cells from post-septic mice exhibit increased expression of Kat2a mRNA; Kat2a is the chromatin modifying enzyme responsible for the addition of the H3K9ac mark. The concurrent increase in both H3K9ac and Kat2a in post-septic CD4+ T cells correlates with the increase in both T_{reg} numbers and functional activity in post-septic mice; in terms of post-septic immunosuppression, this increase in T_{reg} results in increased susceptibility to solid tumor challenge. Taken together, these studies indicate that following severe sepsis, CD4+ T cells exhibit modulations in the epigenetic regulation of gene expression, via changes in histone modifications, and that these changes can have significant effects on the activation, differentiation and effector function of these cells at timepoints post-sepsis.

Clearly, post-septic immunosuppression is a chronic condition, and can manifest itself in survivors of severe sepsis long after the resolution of SIRS and CARS-associated cytokine, chemokine and soluble mediator production. Of particular importance is the ability of post-septic immunosuppression to manifest itself even after the resolution of active suppressive mechanisms, such as with the increase in peripheral IL-10 and TGFβ observed during CARS. Studies of leukocyte function in experimental models of severe sepsis have identified numerous activation deficiencies in post-septic cells, centred on dysregulated cytokine and transcription factor expression. One possible explanation of the maintenance of this immunosuppressive phenotype is via changes in gene expression due to modulations in the epigenetic signatures of post-septic leukocytes. However, these studies have up to now been limited in scope, even within the field of epigenetics, as there remains many other epigenetic mechanisms (such as histone phosphorylation, DNA methylation, miRNA expression, etc) that may also be playing a role in mediating post-septic immunosuppression. In addition, much work remains to be done in correlating the gene regulatory events observed during late-phase CARS in animals with the phenotypes of peripheral blood leukocytes in patients who have recovered from severe sepsis. However, studies in animal models provide experimental data to bolster the findings from epidemiological studies in humans – namely, that post-septic immunosuppression can last far beyond the resolution of CARS, and the long-term survival of severe sepsis patients can be adversely effected by the improper function of the post-septic immune system.

4. Conclusion

Following the onset of severe sepsis, the cellular immune system is tasked with responding to both the septic insult, and any subsequent secondary infections or challenges, while also balancing the need to restrict the same pro-inflammatory responses that are mediating sepsis-induced mortality. The initial molecular mechanisms intended to resolve sepsis-induced mortality – namely, the increase in anti-inflammatory and immune-deviating cytokines observed during CARS – can render the cellular immune system unable to respond to secondary infections. In addition, apoptosis of immune cells during SIRS may

remove those cells from participating in the cytokine storm of severe sepsis, but it reduces the number of functional immune cells able to respond to opportunistic and nosocomial infections. Following recovery from sepsis, the cellular immune system retains many deficiencies in activation and effector function, resulting in persistent immunosuppression that can adversely affect the long-term survival of patients who have recovered from severe sepsis. Ultimately, the cellular immune system is faced with a seemingly unsolvable paradox, in that limiting cellular activation can protect the organism from sepsis-induced mortality, only to leave the door open for mortality based on secondary infections during recovery.

Despite what is known about the nature of cellular immunosuppression following severe sepsis, many questions still remain to be answered. Of particular importance is the apparent link between the severity of the septic response (i.e. the cytokine storm) and the development of post-septic immunosuppression. It appears that the severity of the septic response, presumably as measured by the qualitative severity of the cytokine storm, is directly related to the development of post-septic immunosuppression. However, it is not clearly understood whether the development of immunosuppression is due to the increased level of a specific cytokine, or due to the combined presence of multiple proinflammatory cytokines acting in concert. Clearly, neutralization of individual cytokines during SIRS (for example, by utilizing mice that are genetically deficient) can have a dramatic effect on the sepsis-induced mortality; however, it remains to be seen whether the resulting effects on post-septic immunosuppression are due to the neutralization of the specific proinflammatory cytokine or a generalized effect of reducing the severity of the septic shock. In addition, much work remains to be done on the role of the cytokine storm of SIRS in modulating long-term immunosuppression, in particular the setting of epigenetic marks in post-septic leukocytes. It remains to be seen if the setting of specific epigenetic marks – along with the up- or down-regulation of chromatin modifying enzymes – is mechanistically driven by the cytokine milleu during SIRS.

From a clinical perspective, the persistence of post-septic immunosuppression presents a challenge for both diagnosis and treatment. Treatments aimed at reducing the severity of the acute phase of severe sepsis may help reduce sepsis-induced mortality at the expense of exacerbating CARS-associated mortality. Modulation of epigenetic mechanisms may prove useful for restoring immune function post-sepsis, but the available pharmacological approaches against histone modifications are limited. In addition, the diagnostic tools available to diagnose post-septic immunosuppression remain few, as not all survivors of severe sepsis go on to develop immunosuppressive phenotypes. To date, there are few described markers useful for the diagnosis of post-septic immunosuppression, aside from either functional analysis of peripheral blood leukocytes or, unfortunately, the development of secondary, opportunistic or nosocomial infections in the patient. Ultimately, these challenges associated with the proper diagnosis and treatment of post-septic immunosuppression makes this phenomenon a significant human health concern.

In summary, immune cell dysfunction is an important sequela of severe sepsis. This dysfunction manifests in three distinct phases following the onset of disease: during SIRS as a result of cell apoptosis and exhaustion, during the switch from SIRS to CARS as a result of directed immunosuppression by cells and soluble factors, and for the long term (CARS and beyond) due to cell-intrinsic defects in activation, differentiation and effector function. As a result, survivors of severe sepsis remain susceptible to secondary infections long after recovery from the acute phase of the inflammatory response. Despite the current

understanding of the mechanisms governing post-septic immunosuppression, much remains to be understood concerning the development of this phenomenon and the proper treatments and therapies to recover the proper function of the immune system in post-septic patients.

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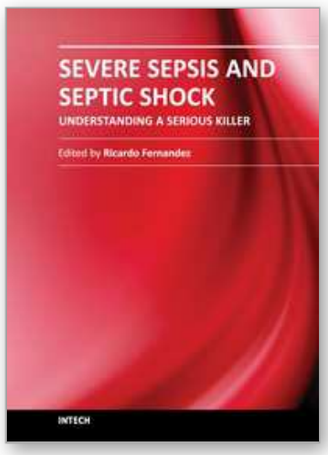

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Despite recent advances in the management of severe sepsis and septic shock, this condition continues to be the leading cause of death worldwide. Some experts usually consider sepsis as one of the most challenging syndromes because of its multiple presentations and the variety of its complications. Various investigators from all over the world got their chance in this book to provide important information regarding this deadly disease . We hope that the efforts of these investigators will result in a useful way to continue with intense work and interest for the care of our patients.

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