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Contribution of anti-inflammatory/immune suppressive processes to the pathology of sepsis

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1. ABSTRACT

Sepsis is the leading cause of death in critically ill patients in the United States. It is associated with enormous expenditures within the health care system and despite substantial human, medical and fiscal resources directed at this clinical entity we have only had a modest effect on the septic patient's long-term survival. However, extensive studies over the last few decades have begun to reveal important pathophysiological processes around which a few promising therapeutic strategies with potential benefits may be derived. It is generally believed, that the body reacts to a septic challenge with an intense hyper-inflammatory response, designed to eliminate the underlying pathogen. However, along with and in response to the intense pro-inflammatory reaction, mechanisms fall into place to counter regulate (control) this initial response, typically resulting in a down regulation of the inflammatory response. This frequently results in dysfunction of various immunological conditions and may result in the inability to ward off the infection and consecutively lead to multiple organ dysfunction, multiple organ failure and death. It is the aberrant development of this anti-inflammatory/ immunosuppressive response, in which it is important to expand our understanding of pathological components to develop potential remedy. Upon this background this review aims to provide an overview on the pathophysiological mechanisms which initiate or maintain the down regulation of the immune response to a septic challenge and which might be a starting point for the development of therapeutic strategies.

2. INTRODUCTION

Although there is great variability on the reported incidence of sepsis it appears that about 750,000 people are affected by sepsis in the United States each year with a mortality of about 28.6% (1, 2). Among more than 6.5 million hospital admissions 2.9% develop a severe form of sepsis (1, 2). The Center for Disease Control attributed about 32,000 deaths in 2001 to septicemia, based on the international classification of disease (3). This identifies septicemia as the tenth leading cause of death in the U.S. (3). An annualized increase of sepsis from 1979 to 2000 of 8.7% resulting in three times as many deaths from sepsis in 2000 when compared to 1979 has been reported (4). Overall, in 60% sepsis is caused by gram-negative bacteria (1, 5).

In the response of the host to a septic challenge, inflammation is thought to play a crucial role (6). In this regard, sepsis has been defined as the systemic inflammatory response that occurs during infection (7). Thus, it seems logical that numerous therapies have been based on the concept of applying anti-inflammatory agents. Among those, corticosteroids (8), anti-endotoxin antibodies (9), tumor necrosis factor antagonists (10, 11), and interleukin-1 receptor antagonists (12) are only a few to mention. However, aggressive maintenance of hemodynamic function (13), intensive insulin therapy (14), low dose corticosteroids (15) and activated protein c (16) are the only remedies that have beneficially altered mortality from sepsis in clinical trials. These agents, with the exception of corticosteroids, are not classic anti-inflammatory agents based on their primary mechanisms of action. The explanations for the failures of the anti-inflammatory approaches have been attributed to several different reasons. First, the animal models used to study many of those anti-inflammatory reagents did not sufficiently emulate the pathophysiological processes leading to sepsis and multiple organ dysfunction (MODS) (17-20). In this regard, the inflammatory reaction provoked, as assessed by circulating cytokine levels, was often a manifold greater than that seen in the clinical setting and often pretreatment approaches were used to study the effect of anti-inflammatory agents (6, 17-19). Second, in the clinical setting multiple risk factors influence the underlying pathophysiological processes that are involved in the development of sepsis and/or MODS. Thus, these may represent confounders in the assessment of the value of a potential therapy. Among those, age, nutrition, gender, disease/injury comorbidity and gene polymorphism are a few to mention (1, 2, 20, 21). Third, it is likely that the development of septic shock and/or MODS is not solely dependent on the initial infectious agent and the evoked hyper-inflammatory response of the host, as patients frequently demonstrate organ failure or succumb in the absence of an infectious agent (22) and as anti-inflammatory agents can especially reveal their therapeutic potential in diseases of clear inflammatory nature such as rheumatoid arthritis and inflammatory bowel disease (23), that said in conditions when no infection is present. However, it is particularly these conditions that become important when trying to explain why and how the capacity to successfully ward off an infectious challenge is reduced in patients with such comorbidity, multiple injuries or in the scenario of a second insult/hit. It is critical to understand on a pathophysiological level what and how the response to a septic stimulus is altered by adverse conditions resulting in a lethargic/anergic immune response in order to establish starting points at which to properly consider boosting the immunologic response.

With this background we attempt to review those mechanisms, which have evolved in the immune system and that are able to counter regulate or resolve an ongoing immune response. By understanding the nature of these players and considering them in the context of the septic patient, we may get some perspective as to how they may drive immune paralysis seen in these critically ill individuals.

In this regard, we will consider four basic mechanisms of immune resolution/counter regulation. First, the release of soluble anti-inflammatory mediators, which in response to a prior release of pro-inflammatory mediators counter regulates the initial innate immune response (Figure 1). Beside soluble receptors for pro-inflammatory agents (i.e. IL-1 receptor antagonist , TNF-Receptor I/II, IL-8 duffy receptor), which primarily have a limited neutralizing effect, cytokines like IL-4, IL-10, IL-13, TGF-beta, glucocorticoids, catecholamines, and prostaglandins of the E series, initiate more profound and multilevel changes in the immune system. They directly affect cytokine production (24-29), apoptosis and proliferation of immune cells (30-37) and interfere with signal transduction pathways (38). The intracellular mode of action for many of these agents appears to involve the induction signaling through janus kinase (JAK), signal transducers and activators of transcription (STAT), phosphatase, the signalling mother against decapentaplegic peptide (SMAD) proteins, certain members of the suppressor of cytokine signalling (SOCS) family as well as alterations in mitogen activated protein kinase (MAPK) family signaling (39, 40).

The second mechanism is closely related to the first mechanism and represents the activation/induction of immune suppressive cell populations. Here, while we separate these for the purpose of discussion, we want the reader to appreciate that this mechanism, like the one before it and those to follow are really intimately interwined aspects of the immune response. The shift towards T-helper type II subclass of CD4 (helper) T-lymphocytes (Th2-cells) following infection and/or inflammation has been extensively studied over the past 10-15 years (Figure 1) (41-48). Th2-cells are defined by their ability to produce anti-inflammatory cytokines such as IL-4, IL-5, IL-6, IL-10 and IL-13 as opposed to pro-inflammatory cytokines like IL-2 and IFN-gamma (cytokines made predominantly by Th1-cells). Both of these cell lineages appear to derive from a Th0 cell. This nomenclature also has been applied to defining CD8 T-lymphocyte sub-populations, i.e., T-cytotoxic-cell-1 and T-cytotoxic-cell-2, which exhibit a similar type of immune suppressive cytokine phenotype (49). In recent years,

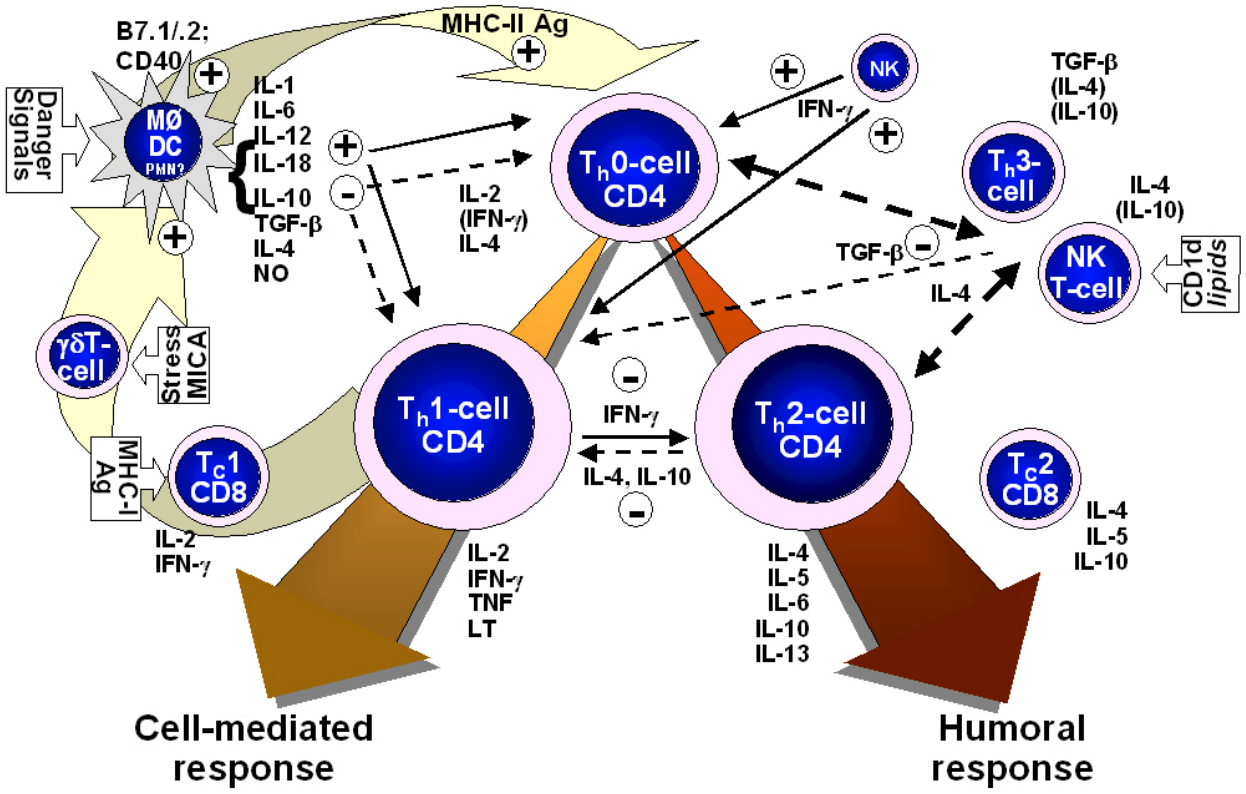

Figure 1. Postulated inter-relationship of T-helper-cells (Th) (Th0-, Th1-, Th2-, Th3-cells), T-regulatory-cells (T-reg), gamma-delta-T-cells, natural-killer-T- (NKT) cell or cytotoxic-T-cell (Tc) -1 and -2 and the cytokines they express in the regulation/development of a competent cell-mediated immune response. See text for detailed description.

along with the development of better agents to phenotype immune cells, additional immune suppressive lymphoid sub-populations have been found and are currently the focus of extensive research. Among those, Th3-cell (linked to gut associated lymphoid tissues and extensive TGF-beta release) (50-55), T-regulatory cells (56, 57) and the Natural-killer-T- (NKT) cell (linked to the liver and intestinal mucosa, producing IL-4 and IL-10) (58-63) are of particular interest. Beside lymphocytes, monocytes/macrophages and dendritic cells, while playing a key role in the innate immune response to infection and/or inflammation can also be significant contributors to the resolution of the inflammatory response and in inducing immune suppression. This in large part is based on the observation that they can be stimulated to be potent sources of many of the anti-inflammatory agents. Recent studies have also shown that even the nature of the materials they phagocytized can drive changes in the array of pro- or anti-inflammatory mediators they release (64, 65). For example, engulfment of apoptotic cells as opposed to necrotic cell debris induces the release of predominantly anti-inflammatory as opposed to pro-inflammatory mediators (64, 65).

The third mechanism mainly involves the loss of cell-cell mediated co-stimulation on lymphocytes and/or antigen presenting cells. In this regard, especially T-cells require a variety of cell surface co-stimulatory molecule ligation to become fully activated/differentiated during the process of antigen presentation (66) (Figure 2). Changes in the expression level of intercellular adhesion molecule-1 (ICAM-1), B7.1, B7.2, and CD40L (67-69) on antigen presenting cells closely regulate the activation/differentiation state of lymphocytes during antigen presentation. Alternatively the activation of inhibitory receptors such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and CD45 within the receptive T-cell can serve to block the activating signal via their capacity to dephosphorylate key components in receptor signaling complex (70-72). Loss of co-stimulation frequently results in the arrest/anergy of T-cell development and programmed cell death of the tolerized cell, leading to the down regulation of growth/differentiation factors release.

The fourth mechanism contributing to resolution of the immune response and possibly to an anergic immunosuppressive status is the activation of programmed cell death in those cells, which are involved in the initial immune response to infection (73-76). In this regard, early and enhanced apoptosis of lymphocytes following infection and/or sepsis is probably one of the most well studied examples of this process in the induction of the immunosuppressive state (77-82). However, the converse of this process, the delay of apoptosis, can also lead to additional tissue damage in some scenarios in the critically ill. This is particularly true with respect to neutrophils in an inflammatory and non

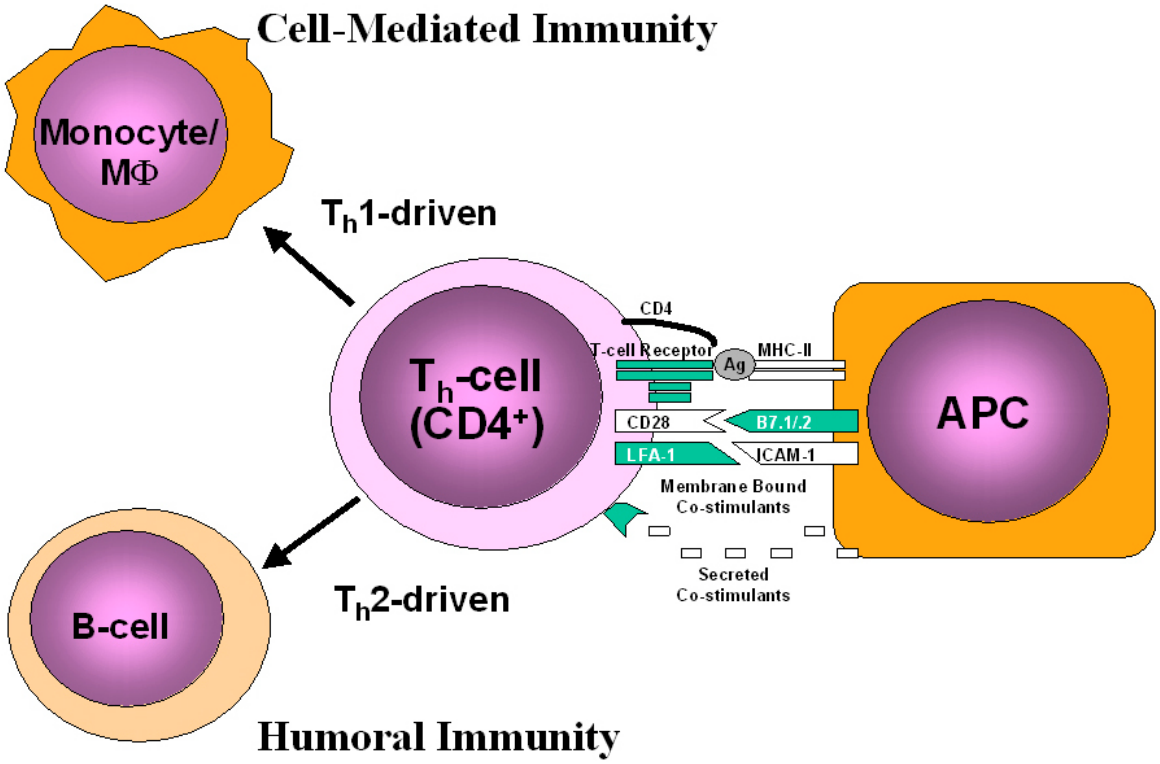

Figure 2. Aspects of the process of T-cell activation in the development of acquired immunity (either cell-mediated and/or humoral) in response to foreign antigen (Ag) presented by an antigen-presenting cell (APC). See text for detailed description.

infectious environment, where the delay of apoptosis they experience is thought to be detrimental to the tissue in which they reside (83-89).

Having now outlined a few possible mechanisms that are involved in the counter regulation of the pro-inflammatory/Th1 driven immune response it seems logical that dysregulation of this secondary response, which restrains the initial immune response, easily could result in a status of anergy and suppression of the immune system. Furthermore, it seems likely that the sometimes non-selective initiation of immunologic events also contributes to unwanted immunosuppressive conditions. In this regard, the enhanced lifespan and activation of neutrophils by inflammation in the absence of infection and the associated tissue damage will be discussed later on. However, it seems of particular importance to shed light on the regulation of these mechanisms outlined above as it is the anti-inflammatory response, which can result in the inability to ward off infectious challenges and which contributes to the subsequent failure of organs and late fatal outcome. That said, we believe it is within those mechanisms that starting-points for the development of beneficial immunomodulatory agents might be provided.

3. RELEASE OF SOLUBLE PRO-INFLAMMATORY/ANTI-INFLAMMATORY MEDIATORS AND THEIR IMMUNOSUPPRESSIVE POTENTIAL

Sepsis or trauma is followed by an increase of circulating pro-inflammatory mediators. It has been suggested, that this early systemic rise in TNF-alpha, IL-1beta and IL-6 might be responsible for the development of organ dysfunction and cell death in response to the insult (90) (Figure 1). In this regard, it has been observed that the elevation of TNF-alpha, IL-1beta and IL-6 early after the onset of sepsis or trauma correlates with morbidity and mortality (91-93). Furthermore, intravenous injection of these mediators generally results in a shock like state (94, 95) and can result in organ dysfunction (96). However, the high doses or extended infusions of these cytokines used in the majority of these studies most likely represent a stimulus that is far beyond the levels detected in response to cecal ligation and puncture (CLP) (97-100) or in septic patients (101). While it has been shown that in sepsis there is a marked early (~1 h peak) elevation in circulating bioactive TNF, followed by IL-6 (~4 thru 24 h) and later by TGF-beta (24 h) (97, 98), these levels of TNF are generally too low to produce shock (101). Also, while the infusion of TNF-alpha or IL-1beta has been shown to result in a shock-like state (94, 95) the reverse logic, that inhibiting them is beneficial, is not necessarily true. For example, pretreatment with antibodies to TNF prior to CLP resulted in increased mortality when compared to sham treatment (100). Furthermore, passive immunization with antibodies against TNF-alpha or IL-1beta did not inhibit sequestration of neutrophils in the lung following CLP (99). These data indicate that it is not simply the presence or absence of pro-inflammatory mediators but also the degree of release and the final amount in circulation that appear to be relevant in driving the immune system towards a immunosuppressive state. Several mediators have been identified as directly or

indirectly affecting mortality following sepsis or shock and in potentially altering the responsiveness of the immune system. In this regard, macrophage-inhibitor factor-1 (102, 103), chemokine migration inhibitory protein-1 (104) and complement fragment 5a (C5a) (105, 106) have been identified as directly affecting organ function and their increase is associated with high mortality. Most recently, high-mobility group box chromosomal protein 1 (HMGB-1) has been described as a late pro-inflammatory mediator in response to infection, injury and inflammation (reviewed in (107)). Whereas there was high lethality associated with the administration of HMGB-1 (108, 109), antibodies against HMGB-1 dramatically reduced mortality in animal models of sepsis (110).

According to Bone *et al*. an initial insult (bacterial, traumatic, thermal) results in a systemic increase of pro-inflammatory as well as anti-inflammatory mediators and subsequently depending on the balance of the two systems can drive either a systemic inflammatory, compensatory anti-inflammatory or mixed antagonistic response (111). However, experience over the last decade suggests that these states do not exclude one another. Moore and colleagues suggested in their global hypothesis for the pathogenesis of post-injury multiple organ failure (MOF) (112), that the systemic inflammatory response syndrome is consecutively followed by the anti-inflammatory response. Furthermore, an extensive inflammatory response accounts for a more severe immunosuppression possibly resulting in MODS and fatal outcome (112). Thus, the question arises as to whether in the end it is the extent of the anti-inflammatory response - influenced by the initial pro-inflammatory response - that drives immune suppression, subsequent organ failure and adverse outcome? In this respect, we initiate our review of the potential immune suppressive mechanisms by a discussion of several typical anti-inflammatory/immunosuppressive agents that have been shown to impose direct and/or indirect effects in experimental and, where data are available, in the clinical setting of sepsis.

3.1. IL-10

IL-10 is an important immunosuppressive player in the anti-inflammatory circuit accompanying/following the pro-inflammatory reaction in response to a pathogen (Figure 1). However, in septic or traumatized patients it can be associated with poor outcome (113-116). IL-10 is produced by T-regulatory cells, which are inducible *in vitro* and *in vivo* under particular conditions of antigenic stimulation (discussed later in this text and reviewed in (117)). B-lymphocytes (24), macrophages, dendritic cells (DC) (118, 119) and non T-regulatory cells (reviewed and referenced in (24, 120)), are other sources of IL-10. IL-10 inhibits the development of Th1 responses and cytokines released by Th1-cells. IL-10 directly inhibits pro-inflammatory cytokine production, e.g. IL-12 production by dendritic cells or macrophages (24, 121). In this regard, a state of decreased IL-12 as opposed to enhanced IL-10 release is also evident in macrophages from mice subjected to CLP or cecal ligation or to implantion of muscle as a source of necrotic tissue (121). Rahim and colleagues have recently shown that besides the known involvement of nuclear-factor-kappa-B (NF-kappa-B) subunits, the effect of IL-10 on IL-12 production in macrophages may also be associated with a decrease in nuclear c-rel levels (122). In synergy with IL-4, IL-10 negatively regulates the survival of dendritic cell (DC)-1 precursor monocytes by inducing their apoptosis (31). In late sepsis the decrease in splenic lymphocyte IL-2 and IFN-gamma release capacity is associated with an increased ability to produce the anti-inflammatory cytokines IL-4 and IL-10 (123). Liu and coworkers have shown that transfecting DCs with IL-10 siRNA is associated with increased CD40 expression and IL-12 production after maturation in those cells (124). Furthermore, silencing of IL-10 in DCs significantly increased allogeneic T-cell proliferation and enhanced Th1 responses by increasing IFN-gamma and decreasing IL-4 production in naïve CD4 T-cells in co culture experiments (124).

With respect to the role of IL-10 in the mortality of CLP, there is some controversy. Work by Hogaboom *et al.* (125) indicates that mice pretreated with polyclonal antibody to IL-10 exhibit a decreased survival to subsequent CLP. Along these lines studies by Minter *et al.* (126) indicate that mice, which have been transiently transfected *in vivo* with an adenovirus containing the human IL-10 gene showed a marked attenuation of the pro-inflammatory response associated with septic challenge in the form of CLP. However, as this represents a pre-treatment approach it still remains to be established what effects this approach may have in the post-treatment setting of shocked and/or septic animals/patients. IL-10 administration following the induction of sepsis in mice is associated with a suppression of the early pro-inflammatory cytokine response but also a suppression of the Th1 lymphokine response and a decrease of the animals' overall survival (127).

Furthermore, our own laboratories results indicate that IL-10 gene deficiency prevents the sepsis-induced depression of Th1 lymphokine production (128). The role of IL-10 in sepsis induced splenic immune suppression *in vitro* was further confirmed by the capacity of anti-IL-10 monoclonal antibody treatment to restore Th1 cytokine productive capacity. Interestingly, Song *et al.* found that *in vivo* treatment of animals with antibody to IL-10 had a salutary effect on animal survival only when administered following the pro-inflammatory/ hyper-dynamic/ hyper-metabolic phase, but not when given during pro-inflammation (128). Thus, our results document not only IL-10's role in septic immune dysfunction, but illustrate that anti-IL-10 administration beyond the initial pro-inflammatory state of polymicrobial sepsis improves survival (128).

Mechanistically, *in vivo* administration of antibodies to IL-10 markedly attenuated the rise in P38 MAPK expression in splenic lymphocytes from septic mice in response to T-cell mitogen while restoring their ability to produce IL-2 and IFN-gamma (38). Thus, MAPK signal transduction appears to be important in mediating the anergic effects of IL-10. More recently, Newton *et al.* have

been able to demonstrate that peritoneal macrophages lacking the CD86 receptor are not able to respond adequately *in vivo* to a septic challenge and that this is associated with an increase in their IL-10 production. This implies a potential immune suppressive role for the co-stimulatory receptor CD86/B7-2 beyond that of simply promoting competent antigen presentation to T-cells (129).

3.2. IL-4

The Th2 cytokine IL-4 is known to regulate Th1- and Th2-cell responsiveness primarily through the activation of the signal transducer and activation of transcription factor-6 (STAT6) pathway (Figure 1). However, while it is suggested that IL-4 may contribute to the suppression of cell-mediated immunity and possibly to fatal outcome seen in sepsis, there has been little information on the role of this agent in the induction of immune suppression seen in sepsis. In this respect, studies in our own laboratory indicated that following the onset of sepsis (123) or following hemorrhagic shock (130), splenocytes isolated from mice exhibited an enhanced capacity to produce IL-4. However, little evidence of this cytokine's presence in shocked/septic mouse circulation was ever revealed. This suggests that the role of IL-4 may be more localized in nature. These studies also did not establish whether IL-4 could actively contribute to the development of immune suppression seen in these mice. In this regard, Song *et al.* (25) observed that when mice were post-treated with neutralizing monoclonal antibody against mouse IL-4 that septic Balb/c mouse splenic lymphoid Th1 cytokine responsiveness could be restored while preventing the enhancement of Th2-cell cytokine release and associated STAT6 phosphorylation. Furthermore, neutralization of IL-4 markedly increased the survival rates in septic animals when given 12hrs post CLP.

Morita and colleagues have recently provided further insight about the mechanisms involved in IL-4 regulated Th1-cell response. They have shown that IL-4-transduced DCs loaded with antigen induced lower T-cell production of IFN-gamma and an increased production of IL-4 (26). Furthermore, they have indicated that the suppression of Th1 mediated and induction of Th2 responses by IL-4-transduced DCs is associated with decreased production of IL-23 (26). The effect of IL-4 on peripheral blood monocyte apoptosis has been addressed by Eslick and colleagues (30). They have demonstrated that IL-4, like IL-10, is able to prevent monocyte apoptosis by upregulation of FLICE-Like Inhibitory Protein (FLIP) and inhibits monocyte pro-inflammatory cytokine production (30). Taken together, these data indicate that the Th2 cytokine IL-4 can contribute to the suppression of cell-mediated immunity and death associated with experimental polymicrobial sepsis.

3.3. TGF-beta

Studies from our laboratory (131) and others (132, 133) have also demonstrated that immunosuppression following shock or sepsis is associated with the systemic release of the anti-inflammatory cytokine TGF-beta. The role of TGF-beta and IL-6 (a direct/indirect inducer of TGF-beta release) as potential anti-inflammatory agents in sepsis, as well as in trauma, has been documented by the work of Miller-Graziano *et al*. (134) and Zhou *et al.* (135). Based on this, one can envision a network of systemic effects mediated by agents that induce the release of IL-6, leading to increased TGF-beta levels that would in turn directly or indirectly induce the suppression of host responses seen during sepsis.

In a series of experiments Williams and coworkers have addressed the role of TGF-beta in infectious lung diseases (136). They have shown that intranasal instillation of plasmid DNA for the TGF-beta gene is beneficial in ameliorating inflammation during *C. neoformans*, *Influenza* and *Respiratory Syncitial Virus* infection by down regulating both, Th1- and Th2-cells (136). However, these effects were associated with a decreased capacity to clear *influenza* and *respiratory syncitial virus*, which resulted in an increase of the replicating pathogen (136). TGF-beta has also shown to decrease E-selectin and IL-8 gene transcription and thus inhibit neutrophil transmigration through endothelial cells (28), potentially inhibiting pathogen clearing by neutrophils. Chen and coworkers have delineated that upon apoptosis of T-cells, TGF-beta is secreted and inhibits pro-inflammatory cytokine production by activated macrophages to foster immune suppression (27). TGF-beta also stifles the expression of Fc receptor for immunoglobulin, inhibits chemokines (i.e. MCP-1), which results in impaired leukocyte trafficking to the site of inflammation and down regulates CD36, a receptor important to the recognition and clearance of apoptotic cells (32, 137-140).

3.4. Prostaglandin E2 (PGE2)

In addition to the anti-inflammatory cytokines, prostaglandins (PGs), particularly PGE2, are important mediators of immuneresolution/immunesuppression. Their production can be induced not only by microbial components such as endotoxin, but also by complement, or by platelet and leukocyte aggregation. There is also evidence that cytokines such as TNF, IL-1, and TGF-beta may also stimulate the release of PGE2 (95, 134). Studies by Ertel *et al*. (141) in rats subjected to CLP indicated both elevated IL-6 and TNF levels in circulation, and a marked increase in plasma PGE2 levels. The anti-inflammatory properties of PGE are by far not restricted to the regulation of leukotrienes. Choudhry *et al.* (142, 143) have reported evidence that prostaglandins appear to mediate lymphocyte dysfunction in a rat sepsis model. We have previously made similar observations following shock where hemorrhage was found to induce such effects by augmenting PGE2 release and lymphocyte responsiveness (130). PGE2 itself has been shown to inhibit proliferation of antigen-stimulated lymphocytes (33) and to suppress cytokine release by mononuclear phagocytes in the lung (29). PGE2 can also exert its effects by inhibiting lymphocyte trafficking through endothelial cell layers (144) via down regulation of ICAM-1, VCAM-1 and ELAM-1, thus preventing them from entering the site of inflammation (145, 146). In general PGE2 *in vivo* seems to result in a inhibition of Th2 differentiation (147), although *in vitro* exposure of lymphocytes to PGE has in part resulted in

divergent effects (reviewed in (148)). In summary, PGE2 not only inhibits pro-inflammatory and anti-inflammatory processes, but also has a marked immunosuppressive effect. That said, the cell/tissue specific distribution of the four subtype PGE receptors (E1-E4) further complicates the situation and broadens the *in vivo* effects of PGE2 (149).

3.5. Nitric Oxide (NO)

Another potentially important immunosuppressive agent, which may contribute to the suppression of lymphocyte-mediated immune response following sepsis, is nitric oxide (NO). Hogaboom *et al.* (125) have shown that NO may play a role in improving survival of female mice subjected to sepsis. Although when a non-specific NO antagonist (L-NAME) was used, they found that such post-treatment also increased the peritoneal exsudate fluid IL-10 levels, though not the capacity of the peritoneal macrophages to make IL-10. However, it is not known if these effects would be comparable if male mice had been used. Chung *et al.* (150) studied the onset of immune suppression in intra-epithelial lymphocytes and found that the NO release by inducible nitric oxide synthase (iNOS) did appear to play a role in the onset of immune hyporesponsiveness in the gut mucosal lymphoid system. Song *et al.* also recently reported that NO derived from iNOS, by an autocrine/paracrine activation loop, may play a role as a contributor to splenic lymphoid dysfunction seen in response to sepsis. In this regard, iNOS deficiency restored IL-2 production but not IFN-gamma production *(151).* Alternatively, it has been reported, that iNOS deficient mice succumb more readily to sepsis *(152).* As iNOS expression is dependent on nuclear-factor-kappa-B (NF-kappa-B) translocation to the nucleus (153-156) as well as activation of p38 MAPK (157, 158), our laboratory set out to determine whether *in vivo* inhibition of NF-kappa-B would also alter septic survival in mice. Using the NF-kappa-B inhibitor, PDTC, as a pretreatment it was found that this increased septic mortality (159), similar to what was seen in iNOS deficient animals (159). In contrast, Wu and colleagues have reported a protective effect in preventing iNOS upregulation in arterioles in sepsis due to protection of impaired arteriolar constriction and pressor responses (160). Thus while NO's role, as derived from iNOS, in sepsis immune dysfunction appears important, its pleiotropic effects make it a difficult therapeutic target.

3.6. Glucocorticoids

Corticosteroids travel easily through cell membranes interacting with cytosolic receptor proteins (161) and affecting transcription in the promoter region of sensitive genes (161, 162). They inhibit transcription factor activator protein-1 (AP-1) (163) and members of the NF-kappa-B-Rel transcription factor family (161, 163-166). In sepsis/septic shock a lack of cortisol or its effect due to adrenal insufficiency occurs in about 10% of severe sepsis patients (15, 167) and a glucocorticoid peripheral resistance syndrome is also often evident (168). However, 50% of patients with severe sepsis typically present increased cortisol levels (15, 167), which have alternatively been linked to an increased risk to succumb (15).

Experimentally, intraperitoneal and intravenous injection of LPS leads to a sudden increase of cortisol in the bloodstream (169). Furthermore, cytokines such as TNF-alpha, IL-1 and IL-6 indirectly increase cortisol via stimulation of the hypothalamic pituitary adrenal axis (170-173). Corticosteroids have a variety of anti-inflammatory properties (reviewed in (174)). Among others, they inhibit pro-inflammatory cytokine production by lymphocytes, macrophages, neutrophils, alveolar macrophages and fibroblasts. They also activate the production of anti-inflammatory cytokines such as IL-10 in macrophages and TGF-beta. INOS, cyclooxygenase (COX)-2, platelet-activating factor (PAF) and macrophage migration inhibitory factor (MIF) production/release are alternatively inhibited. Furthermore, the expression of CD14 on macrophages, as well as endothelial-leukocyte adhesion molecule 1 (ELAM-1), ICAM-1, Lymphocyte-function-antigen-1 (LFA-1) and CD-2 on endothelial cell are also down regulated by corticosteroids (174). Finally, corticosteroids seem to induce apoptosis in immune cells, such as thymocytes and T-lymphoytes.

However, whereas high dose corticosteroid therapy has not shown beneficial effects for septic patients survival over the years, various studies suggest advantageous effects of low dose hydrocortisone therapy in sepsis (reviewed in (175) and (176)). In this regard, low dose hydrocortisone is able to decrease plasma levels of TNF-alpha, C-reactive protein, phospholipase A2 and to increase anti-inflammatory plasma IL-10, IL-1RA and soluble TNF-receptor (177-179). Keh and colleagues recently described the attenuation of the pro-inflammatory and the anti-inflammatory response to sepsis in a double blind, randomized, placebo-controlled, crossover study (180). Besides improvement of hemodynamic parameters, they also observed a decrease in systemic IL-6, IL-8 and soluble E-selectin, a down regulation of CD11b and CD46 on circulating neutrophils and an attenuated release of IL-10 as well as reduced concentrations of both soluble TNF-receptors I and II (180). The assessment of IL-12, IFN-gamma and IL-4 revealed no changes after hydrocortisone treatment, indicating a normal Th1-Th2 response and only subtle differences in the human leukocyte antigen-DR (HLA-DR) expression of monocytes was observed (180). In addition, monocyte phagocytosis was enhanced, granulocyte phagocytosis was slightly depressed and granulocyte respiratory burst was not affected during hydrocortisone treatment (180). These data indicate that low dose hydrocortisone induces a down regulation of the pro-inflammatory as well as anti-inflammatory response to sepsis, while preserving innate immune functions.

Based on these results it appears that exogenous corticosteroids clearly have immunosuppressive effects. These immunosuppressive effects might also be a reason for the failure of high dose corticosteroids in septic patients. Low dose hydrocortisone treatment in septic patients with low cortisol levels however, appears to beneficially blunt pro- and anti-inflammatory immune response without causing immunosuppression (180). This seems to be related to decreased mortality (reviewed in (175) and (176)).

3.7. Sex Hormones

It has also been suggested that the balance of endogenous sex-steroids, especially prolactin, estrogen and testosterone may play an important role in directly or indirectly modifying the host's immune response. In this regard, the impact of gender differences with respect to sepsis has been documented by studies performed by Zellweger *et al.* (181) who found that proestrus female mice survived septic challenge better then age-matched males. Furthermore, a survival advantage in response to hemorrhagic shock, seen in proestrus female animals, appears to be related to not only higher endogenous levels of the sex steroid hormone estrogen (182) but also to elevated levels of prolactin (183). Supplementation of animals with either estrogen or prolactin has been shown to have both immune restorative effects as well as a survival benefit in mice subjected to shock which are subsequently challenged by sepsis (182, 183). Alternatively, endogenous levels of testosterone in male mice appear to contribute to immune suppression in hypovolemic mice (184, 185). The castration of male animals prior to shock or treatment with testosterone antagonists (186) markedly attenuated the loss of immune responsiveness in these animals while providing a survival advantage. Ba and colleagues recently put forward the hypothesis that androgens appear to play an inhibitory role in small intestinal endothelial function (187).

4. POTENTIAL CONTRIBUTION OF LYMPHOID AND/OR PHAGOCYTIC CELL SUB-POPULATIONS TO THE IMMUNE SUPPRESSION SEEN IN SEPSIS.

As mentioned in the introduction, during experimental sepsis in mice there appears to be a distinct shift toward a Th2, or humoral, rather than a Th1, or cell-mediated, immune response (Figure 1). It is now well established that septic patients and animals exhibit enhanced expression of immunosuppressive Th2 cytokines IL-4 and IL-10, with concurrent suppression of Th1 cytokines IL-2 and IFN-gamma (123, 188, 189). However, the mechanisms underpinning the development of this Th1 to Th2 shift are poorly understood. More recently, much effort has been made to identify potential immune cell populations that might contribute to this shift, and whether there are distinct immunosuppressive/anti-inflammatory cell populations underlying both the shift toward Th2 as well as the development of immune dysfunction (Figure 1). That said, the possibility that injury could induce the activation of specific populations of suppressive T-cells is an old concept that was initially proposed by Munster *et al*. (190). Since then, much work has focused on identifying the phenotype(s) of these suppressive T-cells beyond the general CD3/CD4/CD8 markers. A noteworthy sub-population of the CD4+ T helper cell that has begun to draw sepsis researchers scrutiny is the CD25+ T regulatory cell. It has been proposed that this cell population normally exists as a constitutively active immunosuppressive T-cell (56, 57). In view of that, CD4+ CD25+ T regulatory cells have the potential to contribute to the immune dysfunction in sepsis. Studies conducted in our lab and others support this hypothesis, given that the percentage of CD4+ CD25+ T-cells is increased in septic mice and humans. Interestingly, Venet *et al*. (191) showed that this increase was the result of diminished CD4+ CD25- populations and not elevated CD4+ CD25+ cell proliferation, suggesting that CD4+ CD25+ cells, through further activation, may induce the expression of CD25 on their negative counterparts. However, while this establishes an association, further investigation is needed to directly connect CD4+ CD25+ to the pathological alterations and/or immunosuppression observed in sepsis.

NK-T-cells as well as gammadelta-T-cells represent other sub-populations, which may also contribute to the immune suppression observed in polymicrobial sepsis (Figure 1). Gamma-delta-T-cells are considered to be early responders to host defense against a myriad of microbes. Although few studies have investigated the functions of gamma-delta-T-cells in sepsis, initial studies suggest these cells are not involved in the early immune suppression, but may instead play a significant role in re-establishing cell-mediated immune response during the recovery phase (192). NK-T-cells may also contribute to the immune suppression observed in polymicrobial sepsis, as mice depleted of this subset exhibited lower mortality, as well as IL-6 and IL-10 levels in blood and spleen (193). Additional studies are required to further increase our understanding of NK-T and gamma-delta-T-cell involvement in sepsis and its mechanisms.

Natural Killer (NK) cells, a key component in host defense against viral infections, have also been implicated in host response to polymicrobial sepsis (194) (Figure 1). Holub *et al.* observed lower absolute numbers of NK-cells in peripheral blood from septic patients for up to 7 days after onset. In septic animals, the percentage of perforin-positive and IFN-gamma-positive NK-cells has been reported to decrease, suggesting diminished NK-cell function (195). In this regard, depletion of NK-cells corresponded with an inability to clear bacteria early after surgery (196). Though this did not appear to affect the overall survival of septic animals, it does suggest a role for NK-cells in the early host response to invading microbes. Further studies confirmed that NK-cells played a key regulatory role in bacterial clearance via induction of macrophage phagocytosis (197).

Our understanding of the contributions of monocytes/macrophages and dendritic cells has expanded considerably in recent years (Figure 1 and 2). The capacity of macrophages to produce both pro-inflammatory and anti-inflammatory/immunosuppressive mediators is influenced by both timing and the nature of the stimulus. Upon infection, macrophages first produce pro-inflammatory agents, but over time shift toward synthesis of anti-inflammatory agents, which creates an approximate balance of mediators and helps prevent an exacerbated immune response. However, during sepsis macrophages exhibit a compensatory anti-inflammatory response concurrent with the cessation or inability to produce pro-inflammatory mediators. One possible explanation for this altered macrophage response involves the presence of an increased number of apoptotic cell populations. Apoptosis serves as a stimulus for macrophage phagocytosis, and

generally induces anti-inflammatory cytokine production. Another possibility is the induction of an immunosuppressive macrophage phenotype through the p38 MAPK signaling pathway stimulated by NO release from iNOS (151, 198).

Dendritic cells (DC) play a central role in the development of cell-mediated immunity against invading pathogens, and therefore the fate of DCs may provide further insight into the mechanisms of immune suppression in polymicrobial sepsis. Studies from our lab and others reveal a striking decrease in the number of DCs in patients with sepsis and septic animals, predominately mediated by caspase-3-induced apoptosis (199-202). Although apoptosis of DCs from spleen as well as local and distant lymph nodes did not appear to discriminate between immature and mature populations (200, 202), apoptosis of peritoneal DCs appeared to favor the immature population (201). Given these findings, it is conceivable that DC apoptosis, the concomitant loss of antigen presentation and release of pro-inflammatory cytokines from DCs play a crucial role in T-cell impairment and a shift toward Th2 cytokine production.

5. CO-STIMULATORY RECEPTOR/CELL-CELL SIGNALING CHANGES IN SEPSIS: CAUSE OR EFFECT OF IMMUNE DYSFUNCTION IN SEPSIS?

Experimental studies and clinical observations documented that sepsis and many acute severe inflammatory processes result in the appearance of cytokines in the systemic circulation (as discussed earlier), but measuring blood levels of cytokines often does not aid in defining the pro- to anti-inflammatory balance, because serum cytokine levels can change from minute-to-minute and may be very different in adjacent tissue compartments. Thus, as one alternative, the functional status of circulating immunoeffector cells has also been considered in looking for indices that may predict not only the trajectory of the critically ill patients' immune response, but also the onset of organ dysfunction and mortality. In this respect, as a third potential mechanism that might contribute to immune suppression, it is worth reviewing some of the changes that have been seen in key immune cell receptors related to developing organ failure and/or sepsis.

5.1. Human Leukocyte Antigen-DR (HLA-DR)

The monocytes localized at the site of inflammation appear to be one of the predominant participants in defense and repair. HLA-DR, i.e., the major histocompatibility type two antigen (MHC class II), is primarily involved in presenting extracellular foreign antigen on antigen presenting cells, and typically must be expressed at levels greater than 20% to activate a normal cell-meditated immune response (203) (Figure 2). In clinical observations, monocytic HLA-DR expressions have been reported to be significantly decreased in both survivors and nonsurvivors at the onset of severe sepsis. However, where survivors of sepsis showed a continuous recovery of monocytic HLA-DR expression to ≥70% within 10 days, nonsurvivors were characterized by a second decrease in monocytic HLA-DR expression after day 7 or a permanent suppression. Furthermore, the nadir of monocytic HLA-DR expression coincided with the peak of the systemic inflammatory reaction (204, 205). Another study confirmed this early down-regulation of monocytic HLA-DR expression in all infected patients, but found that persistently low expression at day 6 correlated with poor outcome. This alteration in HLA-DR appeared to occurr at a transcriptional level via a decrease in the class II transactivator (CIITA) transcription and could be correlated with increase in cortisol but not IL-10 (206). Other mechanisms of the down-regulation of HLA-DR have also been revealed, such as intracellular sequestration of the HLA-DR molecules. This defect in the process of HLA-DR expression appears to be partially associated with IL-10 (25). Experimental studies looking at models of polymicrobial sepsis also indicate that tissue macrophages derived from the spleen, peritoneum or the liver exhibit a decline in MHC class II expression following the onset of sepsis and that this is associated with a reduced antigen presenting capacity of these cells (129, 207). This same depression in MHC class II has been reported using models of tissue injury (129) and hypotensive shock (208). The mechanism undermining this change is still poorly understood. Interestingly, while a decline in MHC class II expression has been reported in splenic dendritic cells during sepsis, it was not associated with a loss of antigen presenting capacity of these cells (209).

5.2. Co-Stimulatory Factors in Sepsis

5.2.1. Intercellular Adhesion Molecule-1 (ICAM-1)

ICAM-1 serves roles in the activation of either T-cells in response to exotoxin induced shock or macrophages in response to endotoxin induced shock, which can contribute significantly towards a fatal outcome (Figure 2). For example, ICAM-1 deficient mice were found to be resistant to lethal effects of high doses of LPS, which correlated with a significant decrease in neutrophil infiltration in the liver. This protection appears to be related to the loss of ICAM-1 mediated leukocyte-endothelial interactions. ICAM-1 deficient mice were also protected from lethal Staphylococcal enterotoxin B challenge which was associated with a decreased systemic release of inflammatory cytokines. These results suggest that anti-ICAM-1 therapy might be beneficial in both gram-positive and gram-negative sepsis (210). In the CLP model, ICAM-1 deficient mice exhibited a less severe physiological response to sepsis, exhibiting significantly less weight loss and hypothermia. The mortality rate in those mice was significantly lower when compared with WT mice and the degree of leukocyte infiltration was decreased in pulmonary and hepatic tissue, which reduced consequent secondary organ damage, such as acute lung injury (211, 212). Furthermore, saturating concentrations of anti-ICAM-1 monoclonal antibodies reduced lung tissue neutrophils sequestration and migration during *E. coli* challenge (213). Together, these findings document the central role of ICAM-1 in mediating leukocyte-endothelial cell interaction in models of septic organ injury and suggests that this may be the predominant role of ICAM-1 during the early septic pro-inflammatory response. It remains unknown whether changes in ICAM-1 during sepsis play a role in altering its' other effector functions

such as modulating the formation of the antigen presenting cell-T-cell synapse during antigen mediated activation of a cell-mediated immune response.

5.2.2. CD40

CD40 is an integral co-stimulatory molecule in development of adaptive immunity. In this respect, recent studies indicate that CD40-/- deficient mice had delayed death and improved survival after CLP (Figure 2). They also had less remote organ injury as manifested by reduced pulmonary capillary leakage. This protection was associated with reduced IL-6 and IL-10 levels in circulation and bronchoalveolar lavage (214). CD40 has also been demonstrated to be important in the ingestion of bacteria by macrophages (215) and was necessary for IL-12 production by macrophages upon *E. coli* HSP70, but not LPS administration (216). Another interesting observation, which was made from clinical investigations, is that CD40 was found to have an inverse correlation between peak CD40 expression and septic patient survival. Particularly in patients with bacteremia, which exhibited an increase in CD40 expression on peripheral blood monocytes this was found to be a protective phenomenon (217). Our own laboratory's study show that the expression of CD40 was significantly increased in Kupffer cells but not peritoneal or splenic macrophages after sepsis (129). This suggests that the changes in CD40 expression may be markedly effected by the local tissue environment of the septic animal.

5.2.3. B7 & Cytotoxic T-Lymphocyte-Associated Protein-4 (CTLA-4)

Our results showed that the expression of B7-2 (CD86), but not B7-1 (CD80) is significantly decreased on peritoneal macrophages after the onset of sepsis or tissue injury (in the form of cecal ligation alone). The increase of IL-10 production by peritoneal macrophages during sepsis was abolished by CD86 gene deficiency (129) (Figure 2). The expression of CTLA-4 was also increased on peritoneal macrophages but not on splenic adherent cells in CLP mice (201). Alternatively, studies by Ding *et al.* (201) indicate that after sepsis mouse, splenic T-cells (CD3+ cells) express not only an increase in CD40L and CD28 but also in CTLA-4. Interestingly, the results from critically ill patients indicated that CTLA-4 appears to be overexpressed primarily on the circulating CD4+ CD25+ T-cells (205). As these are a potentially important regulatory T-cell population, this suggests a novel mechanism of mediating immune suppression in sepsis, potentially via CTLA-4 activation.

5.3. Contribution of Several Intracellular Signal Pathways

5.3.1. NF-Kappa-B

Nuclear factor-kappa B (NF-Kappa-B) is a cardinal transcription factor in inflammatory response that bridges the stages of recognition and reaction to an inflammatory stimulator and/or infectious agents (Figure 3). CD14, the "universal receptor", can recognize a variety of unrelated foreign biologic materials with the help of host-derived cofactors and then activate an intracellular tyrosine kinase system that eventually activates NF-Kappa-B through its transmembrane signal transducing partners, the toll-like receptors (TLRs) (218, 219). The NF-Kappa-B family is composed of various members, p50 (NF-Kappa-B1), p52 (NF-Kappa-B2), p65 (RelA), RelB, and c-Rel, which can form homo- and/or heterodimers (220). The most studied in sepsis are two dimers, the active form of p65-p50 heterodimer and the non-active form of p50-p50 homodimer. The phosphorylation of the I-Kappa-B-alpha subunit of the NF-Kappa-B complex directs it to be rapidly degraded, and thus frees the dimer to translocate into the nucleus. The activity of NF-Kappa-B can be down-regulated by increased I-Kappa B-alpha, which dissociates the binding of the p65-p50 heterodimer and its responsive elements (221), or by excess production of p50-p50 homodimer (222, 223). That said, defects or alterations in this pathway can lead to incomplete or dysfunctional activity of this pathway that may contribute directly or indirectly to what would appear to be immunesuppression and/or the state of endotoxin (LPS) tolerance/insensitivity seen in the critically ill patients. In this regard, activated NF-Kappa-B can promote the expression of pro-inflammatory cytokines such as TNF-alpha, IL-1beta, and IL-8 plus the pro-inflammatory enzymes iNOS and COX-2, which typically serve to potentiate the innate immune response (224). The results of studies on freshly isolated peripheral blood mononuclear cells (PBMC) of patients with severe sepsis and major trauma indicate that p50 expression is critical in the prognosis of sepsis. The expression of the active p65-p50 heterodimer was significantly reduced for all patients as compared to control donors while the p50-p50 homodimer was reduced in the survivors of sepsis and in patients with trauma. Upon *ex vivo* LPS stimulation, PBMCS of patients with systemic inflammatory response syndrome showed patterns of NF-Kappa-B expression that resembled those seen during LPS tolerance: a global down-regulation of NF-Kappa-B was seen in survivors of sepsis and trauma patients and large amounts of the inactive homodimer were exclusively present in the nonsurvivors (223). It was speculated that this might be a mechanism by which p50 inhibits TNF transcription (225). However, the ratio of p65-p50 to p50-p50 represents only one level at which NF-Kappa-B can down-regulate the inflammatory process, NF-Kappa-B activation can also be regulated at many other stages. I-Kappa-B-alpha synthesis may be stimulated by NF-Kappa-B activation to achieve an autocrine negative feedback loop or indirectly by other anti-inflammatory factors (221, 226, 227). Using the THP-1 cell as a model of endotoxin tolerance, it was shown that I-Kappa-B-alpha could also be the mediator responsible for the reduction of LPS-induced steady state production of the pro-inflammatory cytokine IL-1beta (221). Agonists of the activation of I-Kappa-B-alpha have also been shown to inhibit NF-Kappa-B-induced production of TNF and IL-8 (226). Taken together, these *ex vivo* patient findings and *in vitro* cell study data may help to explain why inhibitors of NF-Kappa-B activation have such divergent effects on *in vivo* models. In those studies of lethal endotoxemia where pretreatment of animals has been carried out with an inhibitor of NF-Kappa-B, like PDTC, some benefit has been seen on survival. This speaks to the classic concept of inhibiting rampant NF-Kappa-B mediated inflammation (228). Interestingly when the septic challenge is modified

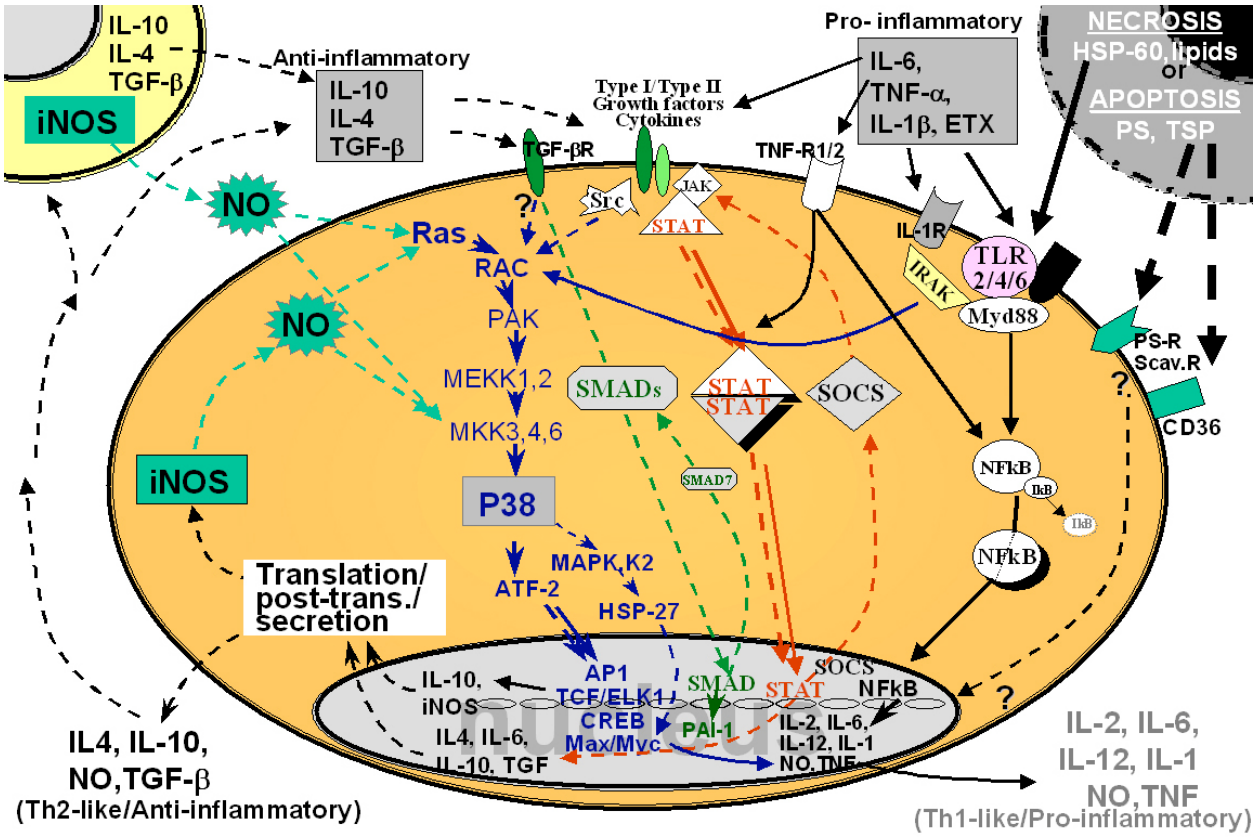

Figure 3. Diagrammatic representation of the various activation signal pathways (——›) induced by presentation of antigen (or mitogenic stimulant) through the T-cell receptor complex (TCR/CD3/CD4), in the presence or absence of co-stimulatory receptor signaling via CD28, various type I/II growth factors, chemokines, as well as a variety of antagonistic signals (-----›) which inhibit activation. Pathways that appear to be involved in transmitting signals, which both stimulate or inhibit cell activation, are indicated by ═══›. For abbreviations please refer to text.

to include an intact infectious agent, as is the case with CLP, the outcome is often worse (159). This speaks to the concept here that a minimal NF-Kappa-B mediated innate immune response is necessary to handle the infectious challenge.

5.3.2 p38 Mitogen-Activated Protein Kinase (MAPK)

MAPKs are involved in all aspects of immune responses, from the initiation of innate immunity, to the development of adaptive immunity, and to resolution of the immune response by apoptosis (Figure 3). Thus it is not surprising that p38 MAPK affects the inflammatory response in sepsis (Figure 3). Studies by Song *et al.* revealed that the expression and activity of p38 MAPK were increased in splenocytes and T-cells during sepsis and that this effect could be reversed by neutralization of IL-10. Alternatively, the expression of IL-10 also seems to be p38 MAPK dependent because the inhibition of p38 MAPK activation suppressed IL-10 expression. In both circumstances, Th1 responsiveness was restored, indicating p38 MAPK may play an important role in immune suppressive response. Another study on iNOS knockout mice supports this hypothesis (38, 151, 229). Furthermore, delayed *in vivo* treatment with SB-203580 an inhibitor of p38 MAPK was found to improve survival after CLP, providing a potential therapeutic target for sepsis (151).

5.3.3. Janus Kinase (JAK)/ Signal Transducers and Activators of Transcription (STAT) and Suppressors of Cytokine Signaling (SOCS)

JAK/STAT proteins provide a direct link between the cytokine receptors and cytokine-induced gene transcription. Several STAT proteins in mammals have a crucial role in host defense (Figure 3). STAT1 and STAT2 mainly mediate the effects of IFNs, STAT3 mediates the effects of gp130 ligands, typically IL-6 and to some extent IL-10, STAT4 and STAT6 mediate the effects of IL-12 and IL-4/IL-13, respectively (230). As mentioned earlier, most of these cytokines play critical roles in the septic process. Our own laboratory's studies indicated evidence of increased activation of IL-4 induced STAT6 associated with the onset of septic immune dysfunction and mortality in the Balb/C mice (25). However, STAT6 deficiency did not completely restore survival although the lymphoid Th1 responsiveness was restored. Both STAT4 and STAT6 gene deficiency provided protection during septic peritonitis (231). STAT6 deficient mice showed increased

levels of IL-12, TNF-alpha, macrophage-derived chemokine (MDC) and an associated enhanced local bacterial clearance. In STAT4 deficient mice, sepsis induced hepatic inflammation and injury were significantly ameliorated without affecting local responses, the hepatic levels of IL-10 and IL-13 were increased while those of MIP-2 and KC were decreased. Renal injury in STAT4 deficient mice during sepsis was also abrogated, which was accompanied by decreased renal levels of MIP-2 and KC without altering IL-10 and IL-13 levels. Together, these data indicate that the balance of type-1 and type-2 cytokine responses contributes to the outcome of sepsis and therefore such signal molecules might be potential targets for modulating the septic response.

Activation of JAK/STAT family members also induce the synthesis of the suppressors of cytokine signaling (SOCS) family of proteins, which inhibit the IL-2, IL-6 and IFN signaling pathways (Figure 3). One could speculate that over expression of such intracellular signaling antagonist could lead to a state of immune cell non-responsiveness to various stimuli. This has in fact been reported for SOCS-1 and SOCS-3 proteins, using models of *in vitro* and *in vivo* endotoxin challenge (232). However, until recently it was not known if the response to polymicrobial sepsis was similar. Using the CLP model, Grutkoski *et al.* observed that SOCS-3 was up-regulated in the spleen, lung, and peritoneal leukocytes [233]. Further only the macrophages and neutrophils appeared to express SOCS-3 in those tissues. This supports the concept that SOCS-3 may be another possible candidate mediator of the immune suppression (233). The result from studies looking at hemorrhagic shock also support this hypothesis (234).

6. MECHANISM OF APOPTOSIS AND THEIR RELEVANCE IN SEPSIS

Over the last decade, various studies have implicated that programmed cell death, or apoptosis, of immune cells appears to contribute to the immune dysfunction and the development of multiple organ failure seen during sepsis (235). The process of apoptosis represents the ability to actively delete selected cell populations in specific tissues (236). While required to fight off a foreign pathogen, for macrophages, lymphocytes and neutrophils their activation often also initiates their subsequent apoptotic death (73-76). The major pathways by which cell death can be initiated are discussed in detail elsewhere (237-252) and are also summarized in Figure 4 to provide context. In brief, three pathways have been described, which appear to drive apoptosis: the extrinsic death receptor pathway (type I cells), the intrinsic (mitochondrial) pathway (type II cells), and the endoplasmic reticulum (ER) or stress-induced pathway. With respect to the death receptor pathway, i.e. extrinsic pathway, apoptosis is initiated upon ligation of soluble (sFasL) or membrane bound (mFasL) Fas ligand (CD95L) and Fas (CD95). TNF-R, TNF-related apoptosis inducing ligand (TRAIL), etc. also belong to the death receptor family (239). Initiator and subsequently effector caspases are recruited and induce programmed cell death via cleavage of DNA in the nucleus. The expression of Fas is regulated in a sophisticated fashion on a variety of cells which determines their susceptibility to Fas induced apoptosis. In this regard, thymocytes, activated B-cells, T-cells, monocytes, macrophages and neutrophils all express the Fas antigen (253). Alternatively, type II cells rely on mitochondria to release cell destruction molecules. Typically, activation of this intrinsic pathway can be initiated by a loss of growth factors such as IL-2, IL-4 or GM-CSF, or the presence of cell stressors such as IL-1, IL-6, steroids and reactive oxygen intermediates, which regulate pro-and anti-apoptotic Bcl-2/Bax/Bad family members (254, 255). Release of pro-apoptotic Bcl-2 family members to the mitochondrial membrane decreases the mitochondrial membrane potential and leads to the release of cytochrome c, Smac/Diablo, and Apaf, which form the apoptosome activating downstream caspases (255). Finally, induction of apoptosis can occur by activating the ER/ stress induced pathway. Oxidant stress, calcium dysregulation and the activation of caspase-12 initiate cleavage of downstream caspase-3 (256).

With our development of a basic understanding of the biology of apoptotic processes and their role in controlling excessive immune reactions (73, 75, 257-259) it has now become apparent that this same process appears to contribute to the pathophysiology of disease states such as HIV immune depression, cancer, autoimmune disorders, neurodegenerative diseases, inflammatory bowel disease and ischemic injury (260-269). However, the question rises whether the programmed cell death of immune cells plays a significant role in the development of the immune paralysis and subsequent morbidity/mortality seen in response to sepsis. In the following section we hope to summarize those findings, which implicate programmed cell death as a contributor to the down regulation and arrest of certain septic immune functions.

6.1. Lymphocytes and Gut Associated Lymphoid Tissues [GALT]

Apoptosis of lymphocytes during sepsis (thymocytes, splenocytes, circulating lymphocytes (B- and T-cells)) are central to the adaptive immune response and rapidly expand in response to cytokines and antigen specific stimulation (Figure 5). While not always considered critical to the early innate/pro-inflammatory septic response, it is clear that the absence of lymphocytes is associated with a decreased ability to survive sepsis (270). It is hypothesized that dysregulation of apoptosis with a loss of thymocytes, splenocytes and/or gut/mucosa associated lymphoid tissue (GALT/MALT) during sepsis might lead to immunosuppression by diminishing the hosts abilities to ward off the ongoing infectious agent (79). In this regard, some of the earliest studies in septic mice indicated that immature T-cells (CD4+ CD8+, CD4- CD8-) of the thymus exhibit apoptotic changes very early (> 4 hrs) in response to sepsis, mainly driven by the initial presence of pro-inflammatory agents such as glucocorticoids and NO (271) or early stimulation by complement (C5a) (272). It has been suggested that thymocyte apoptosis is mainly independent of the extrinsic pathway (78, 273), however there is also evidence, that steroids regulate the expression

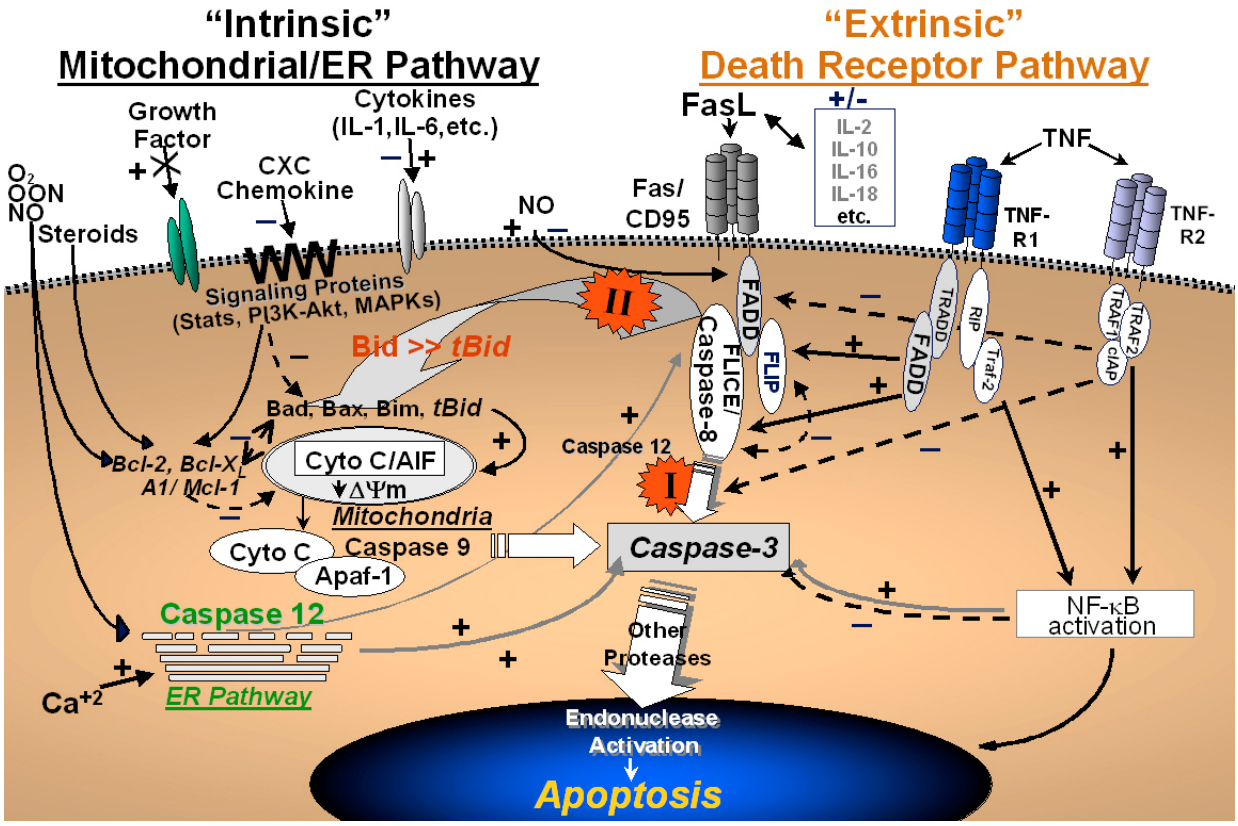

Figure 4. Diagram of some of the key components, mediators and the pathways, which have been implicated in the activation and suppression of immune cell apoptosis. In type I cells apoptosis is mainly induced via activation of the death receptor (extrinsic) pathway, in type II cells via the mitochondrial (intrinsic) pathway. The activation of apoptosis through the endoplasmatic reticulum might be considered as an additional route. Stats (Signal transducers and activators of transcription), PI3K (phosphatidylinositol-3 kinase), MAPKs (mitogen activated protein kinases), C/AIF (cellular apoptosis-inducing factor), ψm (mitochondrial membrane potential), Apaf-1 (apoptosis-activating factor-1), MCL1 (myeloid cell leukemia sequence 1), FADD (Fas-associated death domain), TRADD (TNFR1-associated death domain), TRAF (TNFR associated factor), IAP (inhibitor of apoptosis proteins), RIP (receptor inhibitor protein), FLIP (FADD-like IL-1beta-converting enzyme (FLICE)-inhibitory protein), NF-kappa-B, nuclear factor-kappa-B.

of Fas on thymocytes and thereby inititate apoptosis (274). Death receptor driven apoptosis is more commonly seen in bone marrow and lamina propria B-cells (275), splenic T-cells, intestinal intraepithelial lymphocytes (IELs), and mucosal T- and B-cells of the Peyer's patches (276).

The relevance of lymphocyte apoptosis in septic mice has been demonstrated by a series of studies conducted by Hotchkiss and colleagues. Following CLP, lymphocyte apoptosis was found to be extensively present in thymus and spleen and to a somewhat lesser degree in the lung and gut (77). Whereas we could not confirm the apoptosis of splenocytes during sepsis in our studies (277), we could clearly observe the apoptosis of thymocytes during this event (78). However, more interestingly mice that lack mature B- and T-cells (Rag-1 deficient animals) do not seem to have increased mortality in the first 18 hrs after sepsis when apoptosis is present in most organs (77) but do show an overall decreased survival rate (80). In contrast the transfer of apoptotic lymphocytes has been shown to decrease survival during sepsis via an IFN-gamma dependent mechanism (81). This observation is of particular interest as it suggests that apoptotic cells might participate in the regulation of the Th1-Th2 response during sepsis. In a recent prospective clinical study, Hotchkiss and colleagues reported that sepsis was associated with an increase in apoptotic circulating lymphocytes (CD4 and CD8 T-cells, CD20 B-cells and CD56 NK-cells). Furthermore, the degree of CD3 T-cell apoptosis correlated with the severity of sepsis and this was associated with evidence of activation of both, the extrinsic and intrinsic pathways (82).

As mentioned above IFN-gamma seems to play an important role in mediating the effects of apoptotic lymphocytes on the septic host's immune response. In this regard, the overexpression of Akt in transgenic mice is associated with decreased lymphocyte apoptosis, increased IFN-gamma levels and finally decreased mortality during sepsis (278). The pathological significance of increased lymphocyte apoptosis in sepsis also comes from studies, which report that complete protection against sepsis-

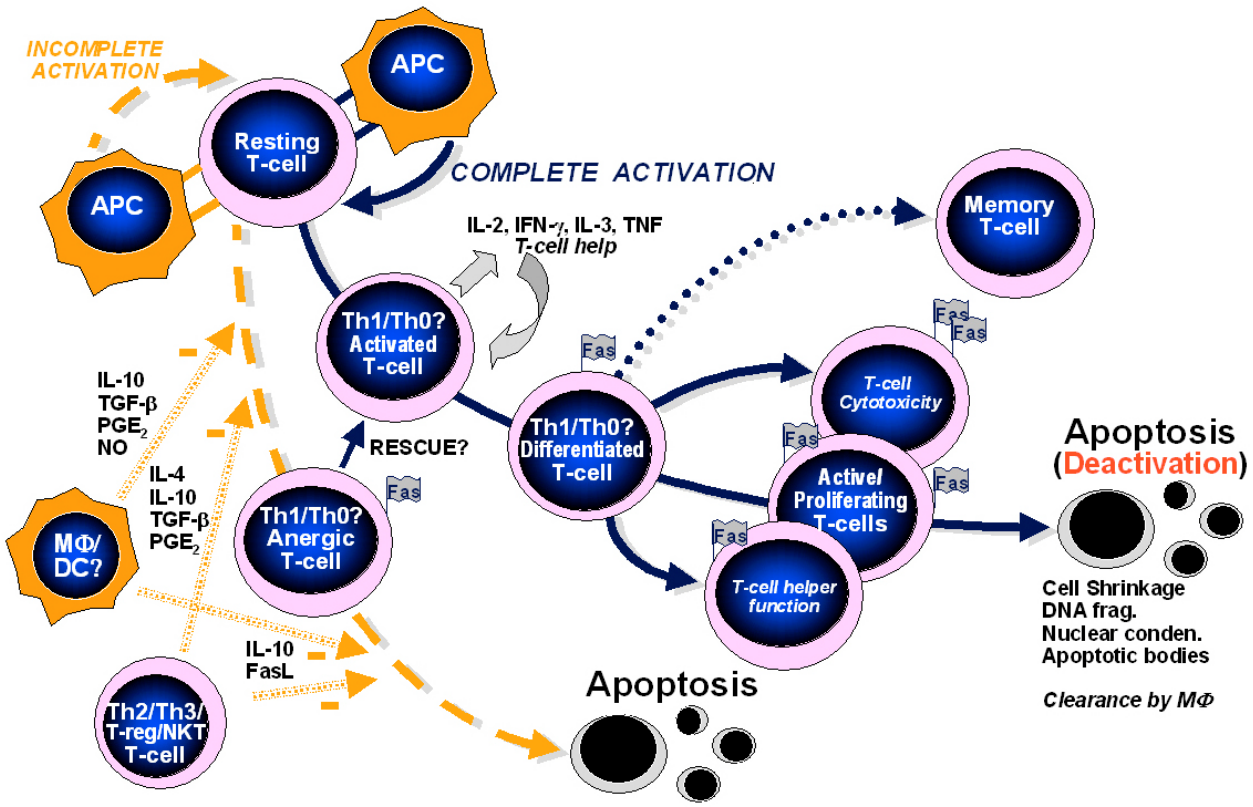

Figure 5. Postulated steps (activation, antiinflammatory induced suppression, maladaptive intracellular signaling, pathologic induction of apoptosis) at which the process of immune cell (T-cell) activation/differentiation might be inhibited/suppressed by various agents leading to a resolution/suppression of the response to foreign antigen in T-lymphocytes following the onset of sepsis.

induced T lymphocyte apoptosis in thymus and spleen and an improved septic survival can be seen in mice which overexpress the antiapoptotic protein Bcl-2 in lymphocytes (80).

Apart from circulating lymphocytes and lymphocytes in spleen and thymus, studies indicate that gut associated lymphoid tissues (GALT), such as the Peyer's Patches, also exhibit increased apoptosis in response to polymicrobial sepsis/CLP, and this appears to be mostly in the B-cell population expressing the Fas receptor (77, 279). Besides the changes in Peyer's Patches, increased apoptosis has also been reported in lymphoid cells lining the small and large intestine in mice 24 hrs post-CLP (77, 273). These changes are also evident in the B-cell subset of the lamina propria. Further assessment of lamina propria mononuclear cell (LPMC) preparations from septic mice *ex vivo* also indicate not only that there are marked increases in B-cell apoptosis but also increases in the % apoptosis in the CD4+ (T-helper cell), CD8+ (cytotoxic T-cell) as well as F4/80+ (macrophages) cells at both 4 (except for CD4+) and 24 hrs (280). Most intriguingly, we have recently shown that the phenotypically distinct intestinal intraepithelial lymphocyte (IEL) population also exhibits changes associated with increased apoptosis (this may be a reflection of localized immune cell activation in response to sepsis as increased cytokine gene expression is also seen in late sepsis) (281). Interestingly, this appears to be a FasL-Fas antigen mediated process independent of TLR-4 sensitivity, which culminates in a marked reduction in late mortality in C3H/HeJ-FasLgld (FasL/TLR4 deficient mice) (281).

Another interesting mechanism by which apoptotic cells regulate the immune response and participate in the counter-regulation of the initial inflammatory response lies in their removal. As eluded to earlier, it has been demonstrated that ingestion of apoptotic cells by macrophages triggers the release of anti-inflammatory mediators, whereas the ingestion of necrotic cells enhances the production of pro-inflammatory mediators (64). In this respect, studies by Hotchkiss *et al.* illustrated (81) that if they adoptively transferred cells, in which apoptosis was induced by *ex vivo* irradiation, to naïve mice prior to polymicrobial sepsis the animals survival was worsened. Alternatively, the transfer of necrotic cells improved survival in septic mice. This supports the hypothesis that apoptotic lymphocytes have the potential to serve as immune suppressants through their clearance during sepsis. Evidence for this mechanism however remains to be established within actual septic mice/patients.

6.2. Neutrophil Apoptosis

Polymorphonuclear granulocytes represent a cell type, whose viable half-life *in vivo* under normal conditions ranges about 1-2 days once released from the bone marrow.

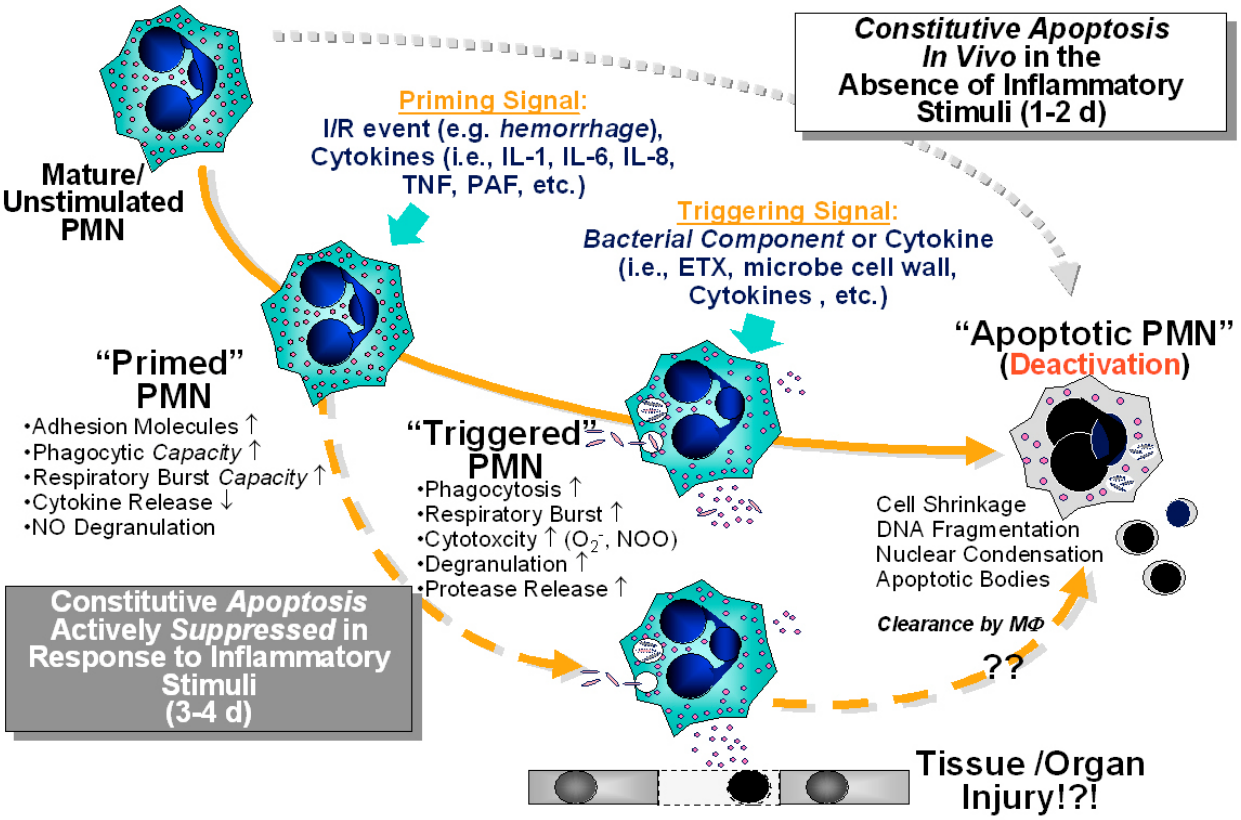

Figure 6. Postulated steps (activation, antiinflammatory induced suppression, maladaptive intracellular signaling, pathologic induction of apoptosis) at which the process of neutrophil activation/differentiation might be inhibited/suppressed by various agents leading to a suppression of the response of neutrophils to a foreign antigen following the onset of sepsis.

In vitro they typically undergo apoptosis within 24 hrs in a constitutive process, which differs from that of lymphocytes and macrophages (Figure 6). Apoptosis in neutrophils can be induced via Fas ligation (282, 283), TNF-alpha (284, 285) or Trail-R (286). However, inhibition of Fas (283, 287) or absence of Fas/FasL *in vivo* (288, 289) fails to prevent constitutive neutrophil apoptosis. Interestingly and in contrast to lymphocytes naïve granulocytes react to an inflammatory stimulus (e.g., LPS, TNF, IL-8, IL-6, IL-1b, GM-CSF) with a delay in apoptosis (85, 88, 89, 290-297). Recent studies suggest that the delayed apoptotic process in circulating neutrophils from septic patients is linked to a decrease in caspase-9 and caspase-3 activity and a prolonged, maintenance of the mitochondrial transmembrane potential (298). Jia and colleagues have recently reported that pre-B cell colony-enhancing factor (PBEF) may be an important regulator of delayed neutrophil apoptosis of clinical and experimental sepsis (299). *In vitro* studies suggest that the postponement of constitutive programmed cell death in neutrophils is initiated by a series of factors such as IL-1beta, IL-8, endotoxin release or regional hypoxia (85, 291, 296, 297, 300-303). In this regard it has been demonstrated, that granulocyte-macrophage colony-stimulating factor and IL-8 delay neutrophil constitutive apoptosis by activating phosphoinositide 3-kinase and extracellular signal-regulated kinase controlled pathways (304). On the other hand, factors such as TNF-alpha and IL-10 (depending on the timing of the exposure) have been shown to increase PMN apoptosis (305-307). The delay in apoptosis is thought to provide sufficient time for PMN to migrate to the site of infection/inflammation and interact with the infectious stimulus (308, 309). However this mechanism does not seem to be solely restricted to the site of infection. In this regard, we have demonstrated that while shock-primed neutrophils exhibit a decreased apoptotic rate, they conversely exhibit an increased capacity to generate oxygen radical species (83). However, if adoptively transferred to a septic environment they migrate unselectively to different organs, e.g. the lung, where they appear to induce tissue damage even without the presence of an infectious agent (83). Depletion of neutrophils ameliorates this form of extrapulmonary acute lung injury. This injury of otherwise healthy tissue by primed neutrophils has been described by a variety of laboratories (84-86, 299, 300, 310). Whereas the hypothesis of decreased apoptosis in activated peripheral blood PMNs from septic patients is largely supported (84-89) there is lack of data supporting their actual ability to produce injury once they have reached their site of interest. In this respect, PMNs in inflammatory sites would be expected to differ from those seen in the blood. Having made this statement, using the CLP model of sepsis alone, we have assessed phagocytes expressing Gr1 (the mouse granulocyte marker)

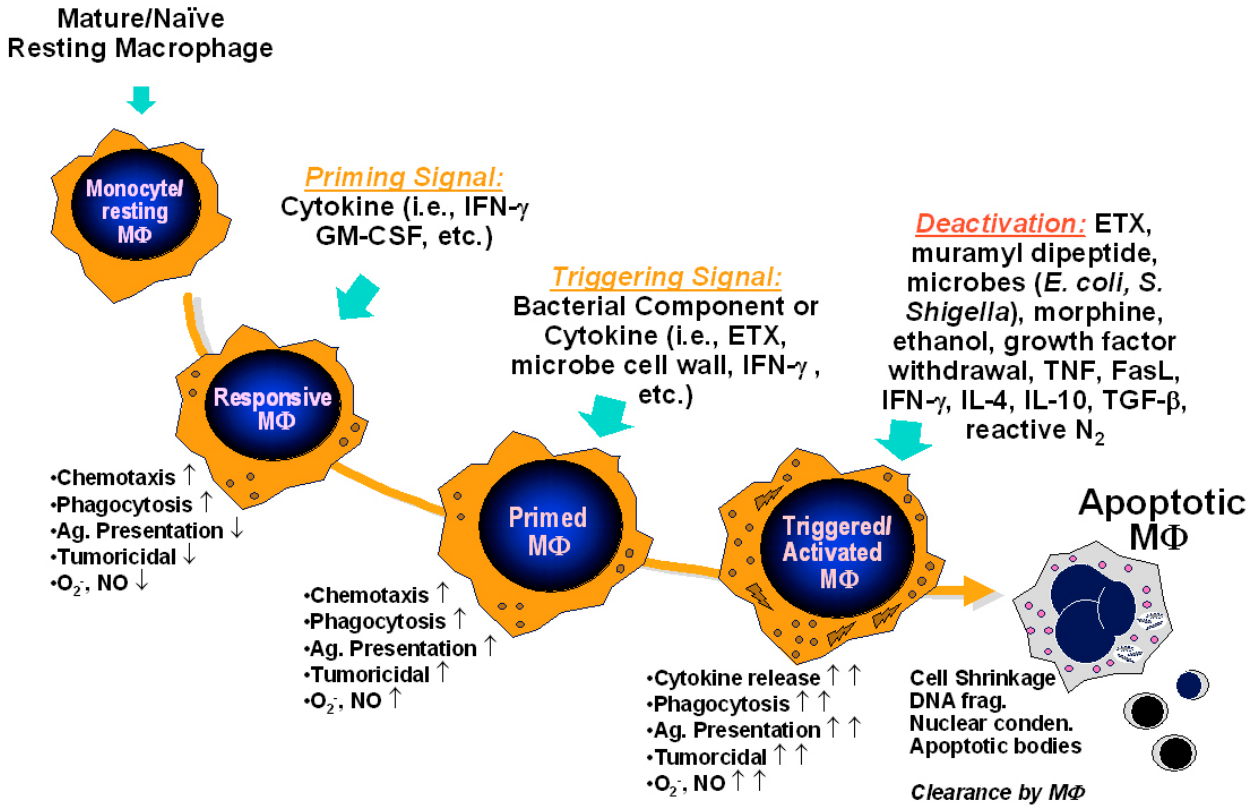

Figure 7. Postulated steps (activation, antiinflammatory induced suppression, maladaptive intracellular signaling, pathologic induction of apoptosis) at which the process of macrophage activation/differentiation might be inhibited/suppressed by various agents leading to the suppression of the response of macrophages to a foreign antigen following the onset of sepsis.

from 3 separate immune compartments along with the extent of apoptosis observed. In agreement with those findings made in patients, decreased apoptosis was seen in CLP mouse blood PMNs (311). Interestingly, no change in % apoptosis was detected in the myelopoetic compartment of the bone marrow (312). However, a marked increase in the % of $Gr1^+$ cells undergoing apoptosis was evident in cells taken from a site of inflammation, the peritoneum of the CLP mice (311). Also, the extent of apoptosis in these cells in the peritoneum appears to be regulated by TNF (311). There is emerging evidence that apoptosis of PMN on a tissue level might be regulated by cytoskeletal molecules, including BIM and BMF (313). Furthermore macrophages seem to be crucially involved in regulating the action of PMN on a tissue level. Studies in neutrophil like HL-60 cell indicate that phosphatidylserine and its oxidized form, which is revealed during the disintegration of the outer cell membrane in the early stage of apoptosis might serve as a signaling molecule for macrophages to start their engulfment, thus limiting the potential danger of the release of the deleterious contents from dying PMN (314). The cleavage of the phosphatidylserine receptor on macrophages, can on the other hand prevent the removal of apoptotic cells and enhance inflammation (315). Macrophages that lack CD44 are not able to engulf neutrophils and thus cannot clear lung inflammation, indicting a crucial role for this cell surface molecule in the recognition or uptake of neutrophils (316). Unfortunately it is yet unknown, whether the loss of the macrophage receptor is significant only to the clearance of apoptotic PMN or other cells in sepsis, or whether its loss effects survival.

6.3. Macrophage/Monocyte Apoptosis

Macrophage apoptosis has been mainly studied under *in vitro* conditions in response to various agents such as ETX (LPS), TNF, IL-1beta, IL-10, IFN-gamma, FasL and NO (Figure 7) (32, 302, 317, 318). Also, while most of the components of the Fas-FasL and TNF pathways are evident, it is less clear that a comparable series of anti-apoptotic gene products are present. In this respect, macrophages, unlike lymphoid cells, typically express lower levels of Bcl-2 and Bcl-X (319) while retaining normal expression of other members of this family such as Bcl-w, Mcl-1 or A1 (320-322).

Unfortunately, there is only limited data on the downstream apoptotic pathways that are activated in macrophages in response to trauma or sepsis. Chung *et al.* found that in peritoneal macrophages from septic mice IL-1beta and IL-18 release/processing as well as caspase-1 activity declines (323). Alternatively, in the same cells, caspase-3 and caspase-9 activity as well as the onset of apoptosis is increased (323). Similarly, alveolar macrophages have been shown to exhibit increased apoptosis in response to low dose pneumococci infection in

bacterimic mice (324). Interestingly, whereas inhibition of caspases clearly reduced apoptosis in alveolar macrophages it also increased the number of bacteria in mice. This suggests the role of alveolar macrophage apoptosis may be important in maintaining tissue homeostasis during pneumonia (324). As already mentioned the engulfment of apoptotic cells by macrophages represents a potent anti-inflammatory/immune suppressive process. It has been described, that phagocytosis of apoptotic cells induces the release of anti-inflammatory mediators (64), such as IL-10 by macrophages, possibly contributing towards a counter-regulatory anti-inflammatory environment (325).

However, the role of apoptosis in regulating the functional responses of macrophages to an inflammatory insult alone is not well documented and has been hardly addressed *in vivo*. This is in part related to the difficulty in clearly establishing whether the apoptotic phagocyte encountered in the septic animal is truly dying from the activation of its own cell suicide process or may just inadvertently appear that way because it is actively involved in clearing apoptotic or dying cells in its environment. Irrespectively, those data thus far concerning the possible induction of apoptotic death in the monocyte/macrophage and/or its development of an anti-inflammatory phenotype through the engulfment of apoptotic cells, provide potential mechanisms of contributing to an overall immune suppressive state in the septic animal (Figure 7).

7. CONCLUSIONS

Infectious challenges and sepsis initiate a primarily beneficial reaction of the host. This reaction is aimed at overcoming the developing pathogenic challenge. Initially a robust pro-inflammatory response, characterized by mediators such as IL-1beta, TNF-alpha and IL-6 followed by late mediators such as HMGB-1 can be observed. This response may be interpreted as an initial activation of the defense mechanisms of the host. The actions of these mediators on the host's immune response are manifold, multilevel, convergent, overlapping and sometimes antipodal in nature. The nature of their expression can be extraordinarily specific for a given pathogen, yet strangely nonspecific, as infectious and non-infectious challenges can exhibit similar responses.

Concomitantly to this initial response, mechanisms come into play, which are designed to shut down the initial activation of the immune system. The mechanisms we have outlined above - while far from being exhaustive - include shifts in systemic and local mediator profiles, changes in proliferation and cell cycle arrest of specific immunoregulatory cells, inhibition of specific co-stimulatory actions, interference with intracellular signal transduction pathways, inhibition or activation of transcription and/or translation processes and programmed cell death of immune cell sub-populations. It is believed that they evolved to protect the host from the consequences of an exaggerated initial inflammatory response. Depending on the nature of the pathogen, the health status of the host, the effectiveness of treatments, and the development over time, these responses will turn out mainly pro-inflammatory, mainly anti-inflammatory or ideally well balanced. Whereas the confinement of the initial pro-inflammatory response by the anti-inflammatory mechanisms described above is at least a theory that might be offered to explain many of these immunological scenarios, the containment of the anti-inflammatory response itself is largely not understood. However, it may well be, that it is the dysregulation of this anti-inflammatory, immunosuppressive response, which may be the critical aspect contributing to overshooting resolution of the initial pro-inflammatory response. This may result in immune paralysis and/or a pathological form of immune suppression. In this state the host is then vulnerable, due to the impaired function of its immune system, and to the untoward effects of opportunistic infectious challenges. It is exactly this scenario of multiple insults that is believed to be such an immense threat to the critical ill patient/ animal. Thus, as therapeutic interventions modulating the initial pro-inflammatory response have largely failed in the clinical arena, it is our hope that as information becomes available from studies directed at understanding the nature of the counter-regulatory process in human and experimental animals, we will be better able to therapeutically interdict in the process to the benefit of the critically ill patient.

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