Leukocyte apoptosis and its significance in sepsis and shock

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Abstract: Sepsis and multiple organ failure continue to be significant problems among trauma, burn, and the critically ill patient population. Thus, a number of laboratories have focused on understanding the role of altered apoptotic cell death in contributing to immune and organ dysfunction seen in sepsis and shock. Immune cells that undergo altered apoptotic changes include neutrophils, macrophages, dendritic cells, as well as various lymphocyte populations. Evidence of epithelial as well as endothelial cell apoptotic changes has also been reported. Although mediators such as steroids, tumor necrosis factor, nitric oxide, C5a, and Fas ligand (FasL) appear to contribute to the apoptotic changes, their effects are tissue- and cell population-selective. As inhibiting Fas-FasL signaling (e.g., gene deficiency, Fas fusion protein, or Fas short interfering RNA administration), caspase inhibition (caspase mimetic peptides), and/or the overexpression of downstream antiapoptotic molecules (e.g., Bcl-2, Akt) improve survival of septic mice, it not only demonstrates the pathological significance of this process but points to novel targets for the treatment of sepsis. *J. Leukoc. Biol.* 78: 325–337; 2005.

Key Words: *mice · human · death-receptor pathway · mitochondrial pathway · immune and organ dysfunction*

INTRODUCTION

Every year, approximately one-third of the reported cases of sepsis associated by trauma, burn, shock, or severe bacterial infection results in death as a result of multiple organ failure (MOF) [1]. Various therapies including anti-tumor necrosis factor (TNF) therapies, antibodies against endotoxin, and anti-interleukin (IL)-1 have been studied; however, the majority of these treatments failed in clinical trials. The major exceptions are activated protein C administration [2], the use of low-dose steroids [3], and insulin therapy directed at maintenance of a blood glucose level at 110 mg/dL blood [2]; however, each of these agents/approaches still only improves septic survival by 10% [4], and their modes of action are poorly understood (and are likely to be not simply anti-inflammatory). Another limitation of the anti-inflammatory agent studies is that much of the preclinical data was often based on lethal bacterial-toxin-based studies, mono-specific intravenous (i.v.) microbial challenge, or pretreatment approaches, which did not replicate the septic patients' status adequately [5]. For this reason, there remains a dire need to better understand the mechanisms of shock and sepsis that underpin the resultant organ failure associated with these states using salient animal models to establish more effective treatments.

Thus, our initial discussion will examine data derived from animal models, such as cecal ligation and puncture (CLP), which more closely resemble the septic patient's response [5]. Sepsis, as produced experimentally by CLP, is characterized by two distinct phases [6–8]. The early, hyperdynamic phase is characterized by increased cardiac output, tissue perfusion, and decreased vascular resistance. The hallmark of this early phase is the proinflammatory state that is mediated primarily by neutrophils, macrophages, and monocytes, which have been stimulated by microbes and/or their toxins. The late, hypodynamic phase (12+ h after CLP) includes decreased tissue and microvascular blood flow, decreased cardiac function, and increased indices of organ injury and dysfunction. In this phase, the immune system exhibits defective antigen presentation, decreased major histocompatibility complex type II (MHC II), loss of delayed-type hypersensitivity response [9, 10], loss of phagocytic function, and decreased T helper cell type 1 (Th1) cytokine release [11]. It is this hyporesponsive phase that may dictate the outcome of sepsis [9, 12], which is why the immune system continues to be a target of research, as dysregulated apoptotic cell death (increased or decreased) is proposed to contribute to the increase in morbidity and mortality that is seen in septic animals and patients [13–15]. It is nonetheless important to appreciate that although CLP is a valuable model of the septic condition, several limitations remain common. Mice cannot be readily or chronically monitored hemodynamically; thus, although acute resuscitation is typically provided, fluid needs over time may not be met. The course of the pathologic and physiologic changes occurs in a more abbreviated manner. Further, antibiotic administration varies among investigators. That said, it's clear that even when antibiotics are still provided, while mortality is altered, it is not fully obligated [16, 17].

MECHANISMS OF APOPTOSIS

Apoptosis is an essential process in which cells are deleted in a controlled manner to limit excessive damage to the surround-

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ing environment. Initially, apoptosis was considered to be a mechanism by which selected cell populations could be actively eliminated from specific tissues during morphogenesis and tissue remodeling; however, it is also thought to have a role in the resolution of the immune response.

A number of studies suggest that dysregulated apoptotic immune cell death may play a role in contributing to the immune dysfunction and MOF observed during sepsis [18–21]. Inducers of apoptosis include steroids, cytokines such as TNF-α, IL-1, and IL-6, Fas ligand (FasL), heat shock, oxygen-free radicals, nitric oxide (NO), and cytotoxic T lymphocytes, which express FasL on their surface as a method of killing a Fas receptor-expressing cell [22]. Apoptotic cell death occurs primarily through three different pathways: the extrinsic death receptor pathway (type I cells), the intrinsic (mitochondrial) pathway (type II cells), and the endoplasmic reticulum (ER) or stress-induced pathway (**Fig. 1**). Here, however, we will only briefly review these apoptotic pathways for context and direct the reader to more specifically detailed reviews of these pathways, which can be found elsewhere [23–26]. Fas antigen [cell differentiation antigen 95 (CD95)] is a cell-surface protein that belongs to the TNF superfamily of membrane receptors, which are responsible for extrinsic apoptotic signaling in type I cells. Fas is expressed on a variety of cell types, including thymocytes, activated B cells, T cells, monocytes, macrophages, neutrophils, as well as on a variety of nonimmune cells in the liver, lung, and heart [27]. When the TNF family receptors, such as Fas, bind to their respective ligands, such as FasL, it causes trimerization and subsequent death-induced signaling complex (DISC) formation, which includes the recruitment of an adaptor molecule also containing a death domain, FADD (Fig. 1), which binds to these activated death domains and to procaspase 8 through death effector domains to form the DISC. The death signal is then transduced from the DISC to a downstream caspase cascade. When procaspase 8 is cleaved and becomes active caspase 8, it can, in turn, cleave and activate downstream effector caspases, such as caspase 3, which cleaves inhibitors of caspase-activated DNase and cleaves DNA in the nucleus [28], leading to apoptosis. The apoptotic pathway can be suppressed by inhibitors such as FLIP, IAP-2, Crm A, and p35 [29–31]. Alternatively, type II cells rely on the mitochondria to release cell destruction molecules, and little DISC is formed. Unlike the extrinsic death receptor pathway, the initiation of the intrinsic pathway is less well-defined. The pathway can be activated by loss of growth factors such as IL-2, IL-4, or granulocyte macrophage-colony stimulating factor, the addition of cytokines such as IL-1 and IL-6, or exogenous stressors such as steroids, reactive oxygen intermediates (ROIs), peroxynitrite, or NO, which in turn, activate pro- or antiapoptotic members of the Bcl-2 family (Fig. 1). Proapoptotic Bcl-2 family members such as t-Bid or Bax are thought to translocate from the cytosol, where they normally exist in a quiescent state, to the mitochondrial membrane, where they act to decrease $\Delta\psi m$. The mitochondrion then releases cytochrome c, Smac/Diablo, and apaf-1, which via apoptosome formation, activate downstream caspases such as caspases 9. These downstream caspases subsequently cause death of the cell. Depending on the balance of the Bcl-2 family members, a dominance of antiapoptotic family members such as Bcl-2 and Bcl-xL can promote survival of the cell [32]. The ER/stress-induced pathway is the least well-understood and appears to involve the activation of caspase 12 by Ca^{2+} and oxidant stress [33, 34] (Fig. 1). This review will attempt to summarize the major observations concerning the pathologic aspects of apoptosis by initially overviewing what is understood in septic animals and/or patients and then briefly considering its expression/role in trauma/shock/sepsis. However, one other point needs to be made when considering the discussion below. For the majority of cases, the studies cited establish evidence of apoptosis using typically two or more methods of detection (DNA-hypoploidy, morphology, DNA laddering, Annexin V, active caspase-3, mitochondrial permeability, and others). This

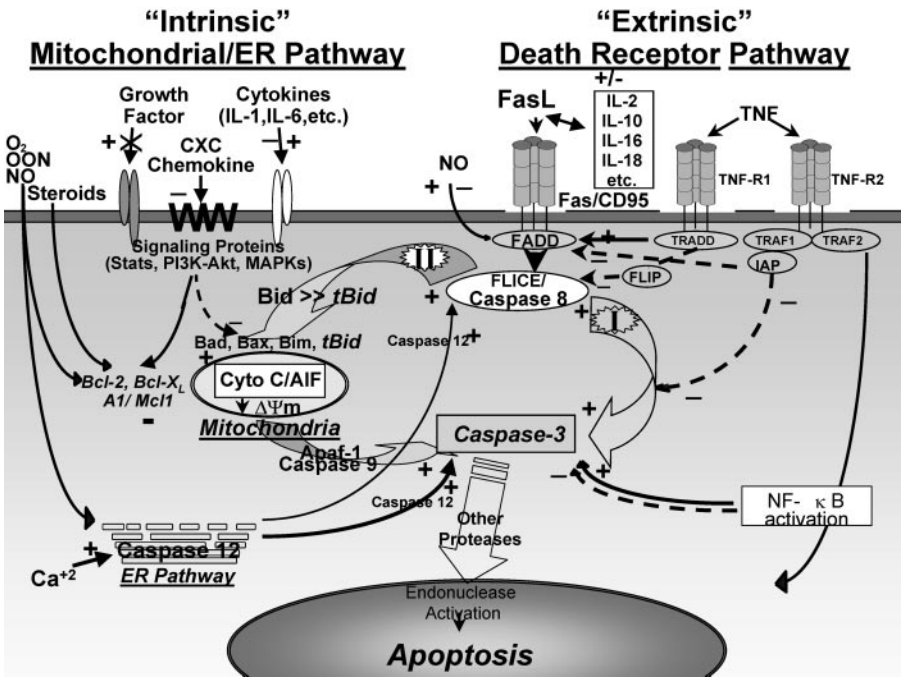

Fig. 1. Summary of general apoptotic signaling pathways as seen through death receptor ligation of TNF receptor (TNFR) or Fas (extrinsic signaling, type I cells) or through activation of the mitochondrial pathway through Bcl-2 family members (intrinsic signaling, type II cells). Stats, Signal transducers and activators of transcription; PI3K, phosphatidylinositol-3 kinase; MAPKs, mitogen-activated protein kinases; C/AIF, cellular apoptosis-inducing factor; $\Delta\psi m$, mitochondrial membrane potential; Apaf-1, apoptosis-activating factor-1; FADD, Fas-associated death domain; TRADD, TNFR1-associated death domain; TRAF, TNFR-associated factor; IAP, inhibitor of apoptosis proteins; FLIP, FADD-like IL-1β-converting enzyme (FLICE)-inhibitory protein; NF-κB, nuclear factor-κB.

is important, as one of the most common methods of detection, the deoxyuridine triphosphate nick-end labeling assay, has a high rate of false-positives [35], thus reducing the potential significance of observations made where this was the sole assay.

APOPTOTIC CHANGES IN SEPSIS

With respect to the cell types that exhibit dysregulated apoptosis after sepsis (**Fig. 2**), the majority of these populations appears to be of lymphoid and to a lesser extent, myeloid immune cell origin, as relatively little overt apoptosis is seen in nonlymphoid or nonimmune organs in the setting of experimental sepsis or clinical specimens [21]. This does not mean apoptotic changes are not evident in these latter tissue or cell types, as we will discuss some of the recent evidence for such alteration, but that it is often more transient in nature and thus, more difficult to detect.

Lymphocyte apoptosis in sepsis

Lymphocytes (B and T cells) are central to the adaptive immune response and rapidly expand in response to cytokines and antigen-specific stimulation. The significance of lymphocytes to the septic animal's survival is documented by the markedly reduced capacity of recombination activating gene (RAG)−/− (lymphocyte-deficient) mice to survive CLP [36]. Normally, apoptosis of lymphocytes is a process to delete autoreactive lymphocytes or to contain/resolve immune cell activation. It is proposed that in the critically ill patient/animal, dysregulated lymphocyte apoptosis in the thymus, spleen, and GALT may lead to immune suppression, leaving the patient vulnerable to subsequent infections or unable to fight existing sepsis, resulting in MOF. In this respect, it was

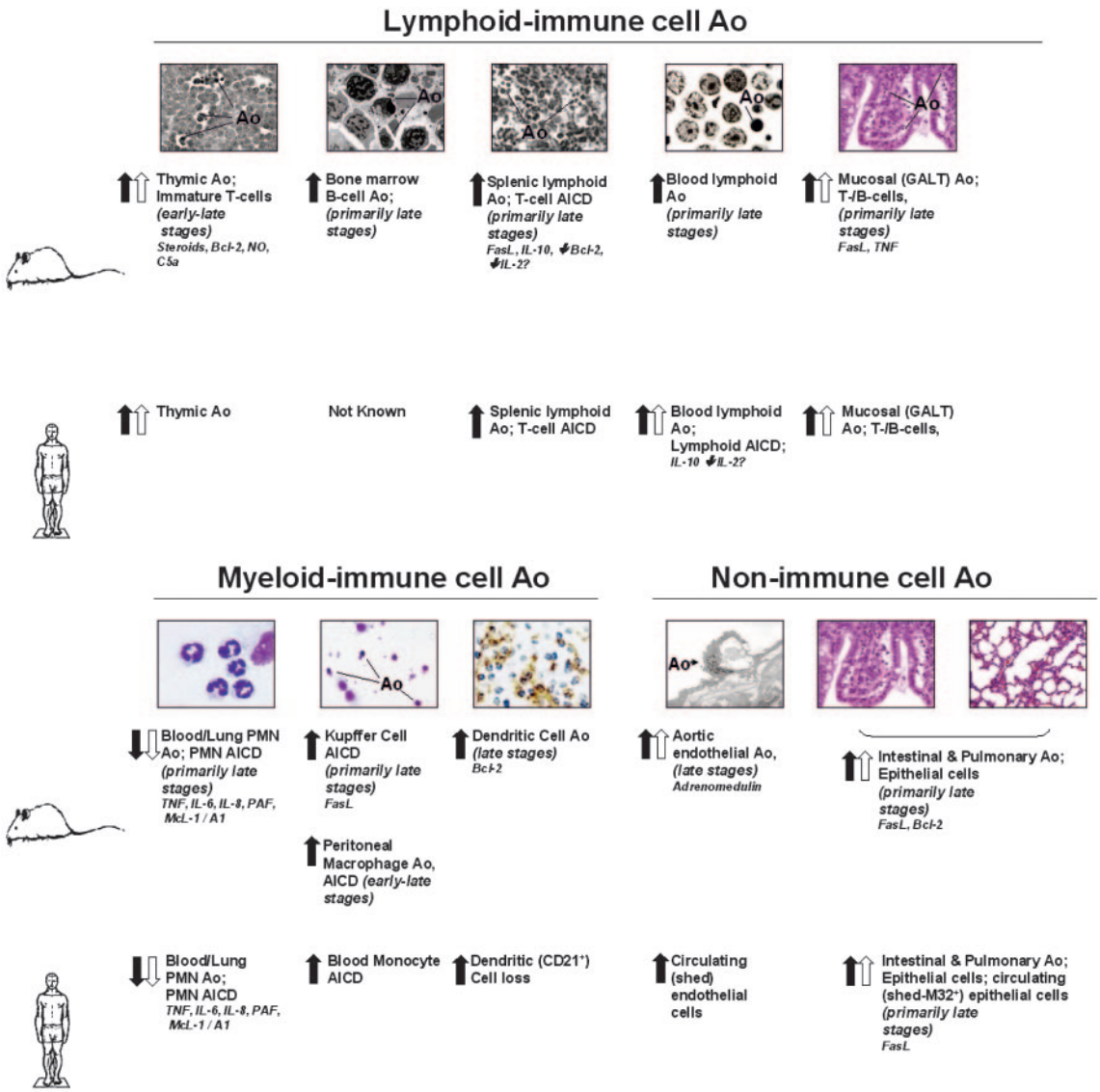

Fig. 2. A summary of the general changes in the levels of apoptosis (Ao) and/or activation-induced cell death (AICD) reported in experimental sepsis (filled arrows) or shock/trauma (open arrows) in mice and septic-critically ill patients, as well as the mediators reported to affect the onset and frequency of apoptosis in various immune as well as nonimmune cell types. GALT, Gut-associated lymphoid tissue; PMN, polymorphonuclear neutrophil; PAF, platelet-activating factor.

initially proposed that an increase in lymphocyte apoptosis as well as the loss of functionally responsive cells might serve to decrease the ability of the septic host to regulate the development of an immune response to opportunistic pathogens, thereby impairing the development of adaptive and innate immune system cross-talk needed to clear the infection [37]. In experimental polymicrobial sepsis, with the exception of the thymus, most lymphoid tissues do not show marked evidence of apoptosis until the late stage (12 h >) after CLP. The thymus, however, shows evidence of apoptosis as early as 4 h after CLP, which for the most part, appears in the immature T cell population ($CD4^+CD8^+$ and $CD8^-CD4^-$ cells). This is explained in part, as thymic apoptosis is primarily a response to early proinflammatory agents such as glucocorticoids and NO [38–40]. It is interesting that this response is largely independent of the effects [39, 41] of endotoxin or death receptors. Another mediator that may contribute directly or indirectly to thymocyte apoptosis is the complement anaphylatoxin, C5a [42]. This is in contrast to the death receptor-driven apoptosis seen in bone marrow and lamina propria B cells [43], splenic T cells, intestinal intraepithelial lymphocytes (IELs), and mucosal T and B cells of the Peyer's patches (PP) [11].

Hotchkiss and co-workers [44, 45] have shown an increase in splenic lymphocyte apoptosis in septic mice, which is associated with an increase in mortality. Lymphocyte apoptosis may be associated with immune dysfunction as a result of decreased proliferation and interferon-γ (IFN-γ) release capability. IFN-γ is a potent macrophage activator and induces a Th1 response [46]. As seen in apoptotic and necrotic splenocyte-adoptive transfer experiments, necrotic and apoptotic cells exercise their effects through variation of IFN-γ levels. Transfer of apoptotic splenocytes retro-orbitally in CLP mice decreased their survival, whereas adoptive transfer of necrotic splenocytes increased splenocyte IFN-γ and in doing so, improved survival. This survival benefit was blocked in IFN-γ-deficient mice or in mice treated with an anti-IFN-γ antibody. These results are interesting, as this adoptive transfer study illustrates the potential impact of apoptotic cells in vivo in sepsis and thus, points at another mechanism (besides loss/death of functional immune cells; **Fig. 3A**) by which immune suppression might be promulgated in the septic animal [47] (Fig. 3B). Alternatively, the inability to clear these dying lymphocytes/cells appropriately (as a result of dysfunction in these cell phagocytic capacities; often seen following sepsis and shock; refs. [48–52]) may allow them to progress to a state of secondary necrosis, producing localized bystander injury in the tissue (Fig. 3C). Such a scenario has been put forward recently by Vandivier et al. [53, 54] as a possible mechanism for tissue inflammation and the enhanced susceptibility to infection seen in cystic fibrosis patients. It remains to be determined whether such defects in macrophage-mediated clearance of apoptotic cells contribute to the changes seen in septic mice.

It has also recently been shown that the serine/threonine kinase Akt [55–57], which is involved in cell proliferation and survival, reduces septic mortality when Akt is overexpressed as a transgene in mice [58]. These mice exhibit a decrease in sepsis-induced (Bcl-2-independent) lymphocyte apoptosis, increased activation markers, and increased IFN-γ [58]. Simi-

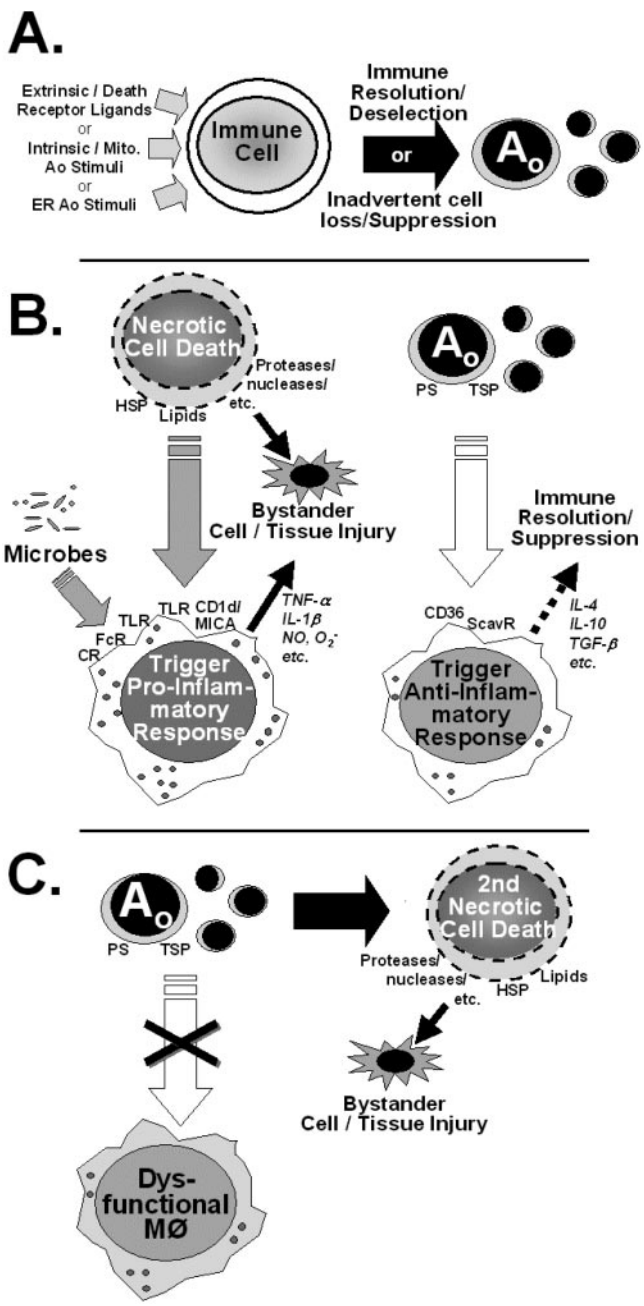

Fig. 3. A depiction of several possible mechanisms of immune suppression. (A) The simple hypothesis (mechanism) that the immune dysfunction observed is a result of advertant/inadvertent apoptotic (Ao) loss of immune cell potential/capacity resultant from extrinsic and/or intrinsic Ao pathway activation. Here, no consideration is made for Ao cell clearence. (B) Alternatively, the effect that clearance of necrotic and/or apoptotic cell materials has on the developing macrophage phenotype (proinflammatory vs. anti-inflammatory/immune-suppressive) is considered when phagocytic function is normal. HSP, Heat shock protein(s); TLR, Toll-like receptor; CD1d/MICA, nonvariant MHC I-like antigen family; FcR, immunoglobulin constant region receptor(s); CR, complement receptor(s); ScavR, scavenger receptor(s), which bind; PS, phosphatidyl serine; TGF-β, transforming growth factor-β. (C) Finally, a scheme in which phagocytic function is compromised, so as to block apoptotic cell clearance, subsequently allowing apoptotic cells to move into secondary necrosis, which in turn, produces bystander tissue injury. TSP, Thrombospondin; Mφ, macrophage(s).

larly, overexpression of antiapoptotic Bcl-2 also decreases lymphocyte apoptosis and in doing so, increases survival [36, 59]. Another possible contributor to splenic lymphocyte damage in the clinical setting is iron. As the critically ill normally exhibit aspects of dysregulated iron metabolism, they are often provided exogenous iron to treat developing sepsis-associated anemia. Studies of septic animals given iron after CLP indicate that iron treatment decreases survival, facilitates bacterial growth, and increases gut epithelial and splenic lymphocyte apoptosis as seen by an increase in active caspase 3 [60]. These effects not only impair the ability of the septic animal to ward off the lethal effects of septic challenge but point at another mechanism, which may contribute to the increasing apoptosis of cells seen. However, with respect to iron therapy in the critically ill, it is also important to appreciate that supplementation in the absence of iron deficiency, such as anemia, can have serious side-effects or even potentiate some pathogen virulence [61, 62].

GALT, such as the PP, also exhibit increased apoptosis in response to polymicrobial sepsis/CLP, mostly in the B cell population expressing the Fas receptor [44, 63]. Hiramatsu et al. [44] reported evidence of increased apoptosis in PP and in lymphoid cells lining the small and large intestine in mice 24 h post-CLP [44]. These changes are also evident in the B cell subset of the lamina propria [41]. In addition, it has been reported that the intestinal IEL population exhibits changes associated with increased apoptosis [41]. This also appears to be a FasL-Fas antigen-mediated process, independent of endotoxin sensitivity, and may be a reflection of localized immune cell activation in response to sepsis. It has been reported that organ damage and mortality associated with sepsis in mouse models are at least in part a result of the activation of the Fas-FasL signaling pathway and not endotoxin, as FasL−/− mice show a marked reduction in septic mortality, which is not seen in the endotoxin-tolerant (TLR4−/−; C3H HeJ) mouse [64]. In this respect, appreciating the inconsistencies in measure-circulating mediators and indices of apoptosis in the critically ill [65–67], studies by De Freitas et al. [68], Papathanassoglou et al. [69], and Roth et al. [70] found that serum levels of proapoptotic factors, such as TNF/TNFR1 and/or Fas/FasL, exhibited a direct correlation with MOF and severe sepsis in patients. These serum levels were even higher in septic patients who died as compared with those septic patients who survived [68, 69].

Dendritic cell apoptosis in sepsis

The dendritic cell is a critical link between the innate and adaptive immunity [71]. Dendritic cells not only migrate to lymphoid organs and stimulate T cells after maturation, but they also play an integral part in lymphocyte apoptosis and immune suppression. Dendritic cells, particularly those that are CD8+ lymphoid-derived, appear to be lost in the spleens of septic patients and mice [36, 72]. This loss of dendritic cells by apoptosis has been seen to occur after $CD3^+$ $CD4^+$ T cell activation [73]. However, the significance and the nature of this apoptotic dendritic cell response in septic animals and patients remain to be fully explored.

Neutrophil and macrophage/monocyte apoptosis in sepsis

With respect to these cells, studies have documented that apoptosis is altered in neutrophils and macrophage/monocytes of clinically septic patients and animals subjected to experimental sepsis; however, to reduce redundancy of the discussion, we have deferred the discussion of these findings to sections below in Changes in the Apoptotic Response Associated with Shock and Trauma.

Nonimmune cell apoptosis in sepsis

In the initial studies, looking at septic apoptosis, many of them focused on the most overt expression of cell death in immune cells, as this was detected easily. Initially, little attention was paid to apoptotic changes in nonimmune tissues, in part, as it was often transient in nature, low in frequency in vivo, and thus, more difficult to detect [21]. However, studies by Coopersmith et al. [74, 75] have indicated that apoptosis contributes to the muscosal epithelial cell loss as well as dysfunction during experimental sepsis, and this can be suppressed by overexpressing Bcl-2 (Fig. 2). With respect to other nonimmune cells, it has been suggested that endothelial cells may be undergoing apoptosis in sepsis; however, this too has been difficult to demonstrate in vivo [76]. The only evidence of endothelial cell apoptosis in septic patients was initially based on the observation that higher levels of "shed" endothelial cells are detected in septic patient blood [77, 78]. However, Zhou et al. [79] have recently documented the occurrence of vascular endothelial cell apoptosis following CLP, which was also associated with decreased levels of antiapoptotic Bcl-2. Other ex vivo/in vitro studies that show endothelial cell survival in sepsis point to antiapoptotic factors. It has been suggested that FLIP may protect endothelial cells against lipopolysaccharide (LPS)-induced apoptosis and suppresses NF-κB activation [31]. With respect to the epithelium, which normally uses apoptosis as a means of cell-turnover, markers of epithelial cell apoptosis have been detected in septic patient blood. The M30 neoantigen, which is a product of Cytokeratin 18 caspase cleavage and release, can be detected via enzyme-linked immunosorbent assay during the process of apoptosis. Therefore, this assay appears to be indicative of epithelial cell apoptosis. Serum levels of the M30 antigen have been observed to be increased significantly in septic patients as compared with trauma patients or healthy controls. Nonsurviving trauma patients (that were not overtly septic), however, did exhibit a significant increase in M30 as compared with surviving trauma patients or healthy controls [80]. Presently, there is still little in vivo evidence suggesting that apoptosis of the liver parenchyma, heart, kidney, or brain occurs in the clinical or experimental setting of sepsis, but it is likely that the apoptotic alterations are not overt and/or may not be the only form of cell death in these tissues.

CHANGES IN THE APOPTOTIC RESPONSE ASSOCIATED WITH SHOCK AND TRAUMA

Besides sepsis, shock (e.g., hemorrhage, ischemia-reperfusion, and others, which are common complications of traumatic

injury), blunt/penetrating, and burn trauma are among the many other different injuries that are associated with the induction of systemic inflammation and subsequent activation of leukocytes, which can induce dysregulated recruitment and/or immune cell activation, resulting in organ damage and MOF. Further, although insults such as shock, blunt tissue injury, ischemia-reperfusion event, and others, as aspects of the initial injury, are often survivable, they too predispose the animal or patient to not only develop organ failure but to reduce the ability to fight subsequent infectious/septic challenge. In this respect, acute lung injury (ALI) and the acute respiratory distress syndrome (ARDS) are the most obvious manifestations of multiple organ dysfunction [81–83]. Thus, with a few exceptions, we have chosen to center the residual discussion of the apoptotic responses contribution to morbidity associated with shock and trauma to ALI.

Altered apoptosis and inflammation also appear to play a key role in the pathogenesis of ARDS. Systemic inflammation is believed to represent a priming stimulus upon which a second, innocuous challenge can induce ALI [84]. However, in ARDS, spillover of local mediators into circulation can also facilitate peripheral organ failure by aggravating the systemic inflammatory response. In addition, dysregulation of programmed cell death (apoptosis) in pulmonary inflammation has been described as a pivotal process regulating the local lung immune response and has been linked to the development of ARDS and/or pulmonary fibrosis [85–88]. Here, immune (particularly neutrophils and macrophages) and nonimmune cell apoptosis, e.g., epithelial and endothelial cells, has been identified to be critically involved in the development of ALI/ARDS [32, 86, 88–96]. Lymphocyte apoptosis also has been shown to contribute indirectly to the immune dysfunction associated with the pathogenesis of ARDS.

Neutrophils and apoptosis in sepsis and shock

The primary role of the neutrophil is to serve as an innate defense against infection by eliminating pathogens. With respect to programmed cell death, these cells typically use their apoptotic machinery in the resolution of inflammation and cell turnover. As they kill pathogens using ROIs and a mixture of lytic enzymes, they can potentially contribute to bystander cell/organ injury. Thus, the normal, constitutive apoptotic death of neutrophils and their clearance is thought to be an important limit on their potential to cause damage. The detrimental effect of these neutrophils on survival has been confirmed using antineutrophil treatments that block lung and liver damage after CLP [97] as well as anti-macrophage-inflammatory protein-2, which blocks neutrophils from entering the peritoneum after CLP [98].

With respect to shock and/or injury, exposure to proinflammatory mediators is thought to serve to prime circulating neutrophils such that a secondary infectious challenge potentiates their targeting to the lung and their ability to induce lung tissue injury [84, 99]. In this regard, our laboratory has demonstrated that neutrophils isolated from animals having undergone hemorrhagic shock (priming) transfer, a similar potential for inducing lung injury when adoptively transferred into naive animals that are then made septic [84, 99]. In this setting, neutrophil in vivo priming was not only associated with an increase in respiratory burst capacity in vitro but also with a decrease in programmed cell death [84, 99]. In ALI, following hemorrhage or endotoxemia, a significant decrease of apoptosis in lung neutrophils has been seen for up to 24 h after the insult [100]. Jimenez et al. [101] found a significant decrease in apoptosis in patients with systemic inflammatory response syndrome (SIRS), and the plasma from these patients was able to suppress apoptosis in neutrophils derived from a control/naïve group. In light of these findings and others [102–104], Jimenez et al. [101] concluded that delayed apoptosis in an inflammatory environment served to fortify neutrophil-mediated killing and in a noninflammatory setting, may contribute to SIRS and ultimately, organ dysfunction and/or failure (Fig. 2). These data suggest that in experimental and clinical settings, suppression of neutrophil apoptosis increases the potential for tissue/organ injury.

It is still not known whether the death receptor or mitochondrial-driven, apoptotic pathway predominates in regulating the cell death of the recruited neutrophils in the shocked and/or septic animal. This delayed neutrophil apoptosis seems to be mainly a result of the activation of antiapoptotic factors. Antiapoptotic members of the Bcl-2 family can inhibit apoptosis through the intrinsic and extrinsic pathways. Although neutrophils do not express Bcl-2 specifically, they do express Bcl-xL, Mcl-1, A1, and Bak [105–111]. A study of neutrophil apoptosis in septic patients indicated that delayed cell death proceeded by activation of NF-κB and then suppression of caspases 9 and 3. The maintenance of the mitochondrial transmembrane potential also appears to be necessary for neutrophil survival following exposure to proinflammatory stimuli [112]. It has been demonstrated that bacterial lipoprotein ligation through TLR2 and CD14 on the surface of the neutrophil may inhibit mitochondrial membrane depolarization, which reduces the level of active caspase 3, thus delaying neutrophil death [30]. This delayed death may be a result of induction of antiapoptotic proteins such as cIAP-2 by endotoxin, which speeds up the degradation of active caspase 3 [29].

Macrophage/monocytes and apoptosis in sepsis and shock

Enhancement of neutrophil apoptosis in ALI has been demonstrated to decrease mortality and ameliorate lung damage [113]. Furthermore, apoptosis is also suggested to play a role in the counter-regulation of the initial inflammatory response after lung injury. In this regard, it has been shown that phagocytosis of apoptotic neutrophils by alveolar macrophages and/or association of alveolar macrophage with apoptotic endothelial cells not only inhibits the release of proinflammatory cytokines [91] from macrophages (Fig. 3B) but also increases their secretion of anti-inflammatory cytokines [114] and growth factors [90], down-regulates FasL expression [115], and initiates T cell apoptosis via c-Myc [115]. Min-Chi Lu et al. [116] have presented findings describing a link between ALI and alveolar macrophage apoptosis in sepsis. As early as 9 h following induction of sepsis via CLP in rats, an increase in macrophage apoptosis was observed. Similar findings were reported in peritoneal and liver macrophages from CLP-induced septic mice by Urbanich and co-workers [117] as well as by Williams et al. [118] and have also been reported in blood monocytes of

septic patients [119]. These studies highlight the role of macrophage as a target of apoptosis and mediator of inflammation via neutrophil/endothelial cell interactions as well as a cause of a dysregulated/dysfunctional immune response to sepsis. However, one limitation to appreciate with respect to the incidence of tissue macrophage apoptosis is that some of these changes may represent an increased role in clearance of apoptotic cells in the local environment, which may make the tissue macrophage look overtly more apoptotic, and it is possible that this is a result of handling a greater amount of apoptotic material.

Nonimmune cell apoptosis in shock

The alveolar epithelium consists of a flat monolayer of cells that provides a critical barrier function and mediates immune responses via the production of cytokines/chemokines. Epithelial cell apoptosis has been reported to be induced by and/or associated with a number of disease states [92, 93, 120–123]. The programmed death of epithelial cells in the lung is frequently seen in ARDS in humans [92, 93, 124] as well as in rodent models of ALI [120, 121] (Fig. 2). Neutrophils have been reported to play a critical role in the induction of pulmonary epithelial cell apoptosis. Serrao and colleagues [122] indicated that neutrophils were capable of inducing lung epithelial cell death via the release of soluble FasL (sFasL) and that this could be blocked by the administration of an inhibitory anti-Fas antibody. Bao et al. [96] demonstrated an attenuation of Fas-mediated epithelial cell apoptosis in the presence of keratinocyte growth factor, a protein associated with wound-healing and lung epithelial cell survival. ALI [120] and ARDS [123] are associated with increased expression of Fas on lung epithelial cells. Along with this increase of membrane-bound Fas, ALI also leads to an increase of sFasL as well as sFas [123]. Following LPS-induced lung injury in mice, epithelial cells display an augmented expression of Fas [95], along with an increased migration of FasL, expressing inflammatory cells into the alveolar space [120]. In addition, high doses of sFasL have been demonstrated to induce lung epithelial apoptosis [125].

Although apoptosis of epithelial cells in the lung seems to be an important factor in the development of ALI, there is again less data describing the role of endothelial cells. As mentioned earlier, endothelial cells represent the barrier between the intravascular space and the tissues supplied by the vascular endothelium. These cells play an important role in neutrophil migration, blood flow regulation, edema, and healing. As such, the integrity of the endothelium is critical, and injury or dysfunction (apoptosis) of endothelial cells is associated with a number of disease states including ARDS [126]. Hotchkiss and colleagues [94] found that in *Pseudomonas aeruginosa*-induced pneumonia, pulmonary endothelial cells rarely underwent apoptosis. However, they suggest that as a result of technical limitations, the role of endothelial cells undergoing apoptosis under these circumstances is not yet clear [127]. In vitro data from Pohlman and Harlan [128] and Hu et al. [129] supported the findings of Hotchkiss et al. [94]. They found that *Escherichia coli* LPS was not able to induce endothelial cell apoptosis. Alternatively, endothelial cell apoptosis has been observed in mice following intraperitoneal challenge with *Salmonella typhimurium* LPS [95, 130]. Fujita et al. [131] and Kawasaki and co-workers [132] describe endothelial cell apoptosis in mice with lung injury following i.v. administration of *E. coli* LPS. Most recently, Lu et al. [133] have shown that in experimental shock, evidence of endothelial cell apoptosis can be seen and thus, may be mediated by a factor in shock lymph (Fig. 2).

Lymphoid apoptosis in shock

Thus far, we have discussed the deleterious effects of apoptosis in the innate immune cell population with respect to lung injury. As mentioned earlier, T cells, natural killer (NK) cells, and B cells contribute to the adaptive immune response to inflammation and are critical in maintaining host defense. Lymphocyte apoptosis serves to maintain homeostasis, eliminating autoreactive cells and allowing potent responses to pathogenic challenges. Yet, severe lymphocyte apoptosis can lead to immunosuppression and contribute to the risk of a secondary, opportunistic infection, as observed in the critically injured and AIDS patients [134] (Fig. 2). Bacterial- and viral-related respiratory failure is common in immunocompromised patients lacking functional lymphocytes [134]. Hao et al. [135] described Fas-FasL interactions as causal in the loss of T and B cells in secondary lymphoid organs. They found that loss of Fas activation in mice produced lymphoproliferative diseases; yet, if this state were coupled with an inflammatory environment, over time, a decrease in T and B cells was observed, and this decrease was followed by pulmonary failure [135]. In a clinical setting, Lenardo et al. [136] observed a state of immunodeficiency in patients with defective, Fas-induced peripheral blood lymphocyte apoptosis. A mutation in caspase 8 left these patients susceptible to herpes virus infections, as well as numerous pulmonary infections [136]. Fas-related apoptosis in lymphocytes has been studied in other organs as well following experimental shock. Xu et al. [137] identified an increase in B cell apoptosis in mucosal PP from mice following trauma and hemorrhage, thus affecting a gut-immune response. Subsequently, Hotchkiss et al. [138] identified the presence of transient increases in intestinal epithelial and lymphoid apoptosis in the intestine of trauma patients undergoing surgery following injury. The thymus is the primary site of T cell development and release. Following trauma/hemorrhage in mice, Xu et al. [139] also observed an increase in thymic apoptosis and an associated lack of functional T cells, as well as immunosuppression in these animals. In this respect, Middleton et al. [140] made a similar observation concerning increased thymocyte apoptosis in trauma patients, who succumbed more than 3 h following their initial trauma. Regarding circulating blood lymphocytes, increased susceptibility of T cells to activation-induced apoptosis has been reported by Teodorczyk-Injeyan et al. [141], Oka et al. [142], Schroeder et al. [143], Pellegrini et al. [144], Roth et al. [70], and Delogu et al. [145] (Fig. 2).

Although these studies above have looked at T cells in divergent tissue sites, they mainly characterized changes in the classic $CD4^+$ versus $CD8^+$ lymphocyte subpopulations. As our knowledge of adaptive immune response has grown, the role of unique lymphoid populations, such as $CD4^+CD25^+$ regulatory T cells, NK T cells, $\gamma\Delta$-T cells, and others, has become evident. However, until recently [146–149], the contribution of these lymphoid cell populations had been largely under-appre-

ciated in shock and sepsis. Unfortunately, although it is clear that these cell populations are susceptible to apoptosis [150–154], no data are as yet available, which speaks to the extent to which they are targeted and/or serve as inducers of the apoptotic changes seen in these states.

ANTIAPOPTOTIC TREATMENTS IMPROVE SEPTIC OUTCOME

As our understanding of the mechanisms that underpin the apoptotic process has grown, along with our recognition of program cell deaths, possible contributions to not only sepsis and shock but to interest in other diverse diseases, such as cancer, Alzheimer's, stroke, and AIDS, have grown from a basic and clinical perspective in the development of agents that can alter the process. In this regard, several inhibitory strategies have been applied in animal models, described below, which may point to novel, therapeutic approaches to shock and sepsis-induced apoptotic changes in the clinic (**Table 1**).

Pan-caspase inhibitors have been used in experimental mice and have improved septic survival by 40–45% by blocking apoptosis downstream. The functional benefit of caspase inhibitors was thought to be that they prevent lymphocyte apoptosis, as caspase inhibitors given to mice RAG−/− had no benefit [156]. However, because of the potential toxicities of caspase inhibitors and the lack of specificity at doses needed for inhibition [156, 159, 160], they are not currently being used clinically. Alternatively, recently, studies have shown that the oral use of antiretroviral protease inhibitors that prevent apoptosis in vitro is also able to improve the survival of septic mice to 67%, and specifically prevent lymphocyte apoptosis [112]. With respect to specific targets in the Fas signaling pathway, we have shown that using FasL gene-deficient mice [64], Fas receptor fusion protein (FasFP; Amgen Inc., Thousand Oaks, CA) [18] and more recently, Fas and caspase 8 short interfering RNA (siRNA) reduce mortality from experimental sepsis as produced by CLP. The systemic effects of these treatments or gene deficiencies are unknown; however, there is a possibility that suppressing apoptosis prevents the development of multiple organ injury. In this respect, the survival benefit produced by the hydrodynamic administration of Fas siRNA, which is thought to localize primarily in the liver, provides further preliminary data suggesting that blocking apoptosis in the liver may protect against subsequent multiple organ dysfunction in this model [155, 161]. It is unknown how directly or indirectly blocking Fas-mediated apoptosis would improve hepatic function. However, giving FasFP 12 h post-CLP also produces a septic survival benefit, which was associated with a reduction in the increase in activation induced Kupffer cells apoptosis [18]. Blockade of this Fas-FasL signaling by FasFP has proven to prevent hepatic injury by attenuating the plasma levels of alanine aminotransferase and aspartate aminotransferase and restoring total hepatic, intestinal, and cardiac blood flow during sepsis [19]. Studies by Wang and Chaudry [162] have shown that in CLP, decreased tissue and microvascular blood flow are not seen until the hypodynamic stage of sepsis but occur at 12+ h after the onset of sepsis [163]. It is interesting that FasFP, given at 12 h post-CLP but not earlier (0 h), was the only time-point at which it was shown to have a positive effect [18]. Based on this, one might speculate that pathological events that contribute to these septic aberrations in organ damage/blood flow develop late following onset, leaving them as targets for delayed FasFP treatment. However, whether the effects of the administration of FasFP after sepsis were a result of a direct/indirect effect on the vasculature or some other local cell population is not known.

Fas siRNA, given only 30 min after CLP, indicated that it could improve survival by 50%, whereas FasFP, when given immediately after CLP, did not. This difference may relate to the biology of siRNA function and its ability to suppress gene expression for a prolonged period of time. siRNA, given hydrodynamically in vivo, has been shown to maintain its suppressive effect up to 10 days following i.v. injection [155, 161, 164], so in the case of our sepsis treatment, it was still as fully

TABLE 1. Treatment Approaches That Change Survival Benefit in Experimental Sepsis

Treatment	Effect
Target intrinsic and/or extrinsic pathway:	
Fas fusion protein [18]	Given 12 h after onset of sepsis prevents apoptosis and hepatic injury - ↑ *survival*
Fas siRNA [155]	Prevent apoptosis in the liver, may suppress the development of MOF - ↑ *survival*
Caspase inhibitors [156]	Prevent lymphocyte apoptosis - ↑ *survival*
Bcl-2 overexpression [36, 75]	Inhibits activation of downstream effector caspases, preventing dysregulated apoptosis - ↑ *survival*
p53 deficiency [157]	Prevent thymic but not splenic septic apoptosis - ↓ *survival*
Other agents reported to affect apoptotic pathway:	
Adrenomedullin [79, 158]	Vasodilator, increases microvascular blood flow and cardiac output - ↑ *survival*
anti-C5a [42]	Reduces thymic apoptosis - ↑ *survival*
Protease inhibitors [112]	May limit the overabundant, proteolytic response with respect to neutrophil influx and tissue injury
Akt overexpression [58]	Inhibits activation/interaction of proapoptotic Bcl family members with mitochondria - ↑ *survival*
Inducible NO synthase (iNOS) gene deficiency [40]	Inhibit thymic apoptosis in sepsis - ↓ *survival*

functional at the 12-h time-point as FasFP. Because of its prolonged and yet nonpermanent maintenance of gene suppression, it seems plausible that silencing Fas or other members of the proapoptotic pathway for that matter may be accomplished with siRNA as a treatment approach. Also, as the mechanism by which siRNA enters cells is still under investigation, a more reasonable delivery system would have to be designed that does not use a large volume but also effectively delivers the siRNA to target cells to achieve quick uptake, especially in trauma situations such as those in the emergency room and intensive care unit. Here also, although we observed no indication of inflammation related to the hydrodynamic delivery of control or gene-specific siRNAs, studies would be needed to establish the inflammatory potential of such approaches. Further, although this form of nucleic acid delivery was thought to be liver-specific, initially, it is clear that a number of tissues and cells take up hydrodynamically administered siRNA. Thus, from a pathological and therapeutic approach, more information about cell targeting is needed, as well as better methods for directing these agents to specific sites/cells.

SUMMARY

The data from the past 10 years have provided us with a better understanding of the role of apoptosis in sepsis and shock. More specifically, it is becoming clearer how dysregulated apoptosis of specific immune cells alters their response and their ability to ward off the lethal effects of sepsis. This appears to correlate directly with septic outcome and survival in the experimental setting. It is important that many of these same changes appear to be present in the clinical setting. Finally, it is evident in the experimental setting that the intrinsic/mitochondrial and extrinsic/death receptor-mediated processes may be contributing to the immune hyporesponsiveness that is seen. Future studies will hopefully provide a clearer picture of the nature of these pathological interactions so as to provide novel, therapeutic targets for the treatment of this condition.

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