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The Apoptotic Pathway as a Therapeutic Target in Sepsis

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Abstract

Recent research has yielded many interesting and potentially important therapeutic targets in sepsis. Specifically, the effects of antagonistic anti-cytokine therapies (tumor necrosis factor- α [TNF- α], interleukin-1 [IL-1]) and anti-endotoxin strategies utilizing antibodies against endotoxin or endotoxin receptor/carrier molecules (anti-CD14 or anti-LPS-binding protein) have been studied. Unfortunately, these approaches often failed clinically, and in many cases, the efficacy of these treatments was dependent on the severity of sepsis. Recently, clinical trials using insulin to lock blood glucose levels and activated protein C treatment have showed that while they provided some survival benefit, their efficacy does not appear to be predicated solely upon anti-inflammatory effects. Here, we will review work done in animal models of polymicrobial sepsis and clinical findings that support the hypothesis that apoptosis in the immune system is a pathologic event in sepsis that can be a therapeutic target. In this respect, experimental studies looking at the septic animal suggest that loss of lymphocytes during sepsis may be due to dysregulated apoptosis and that this appears to be brought on by a variety of mediators effecting 'intrinsic' as well as 'extrinsic' cell death pathways. From a therapeutic perspective this has provided a number of novel targets for clinically successful current, as well as future therapies, such as caspases (caspase inhibition/protease inhibition), pro-apoptotic protein-expression (via administration and/or over-expression of Bcl-2) and the death receptor family Fas-FasL (via FasFP [fas fusion protein] and the application of siRNA against a number pro-apoptotic factors).

Keywords

Sepsis; mice; human; apoptosis; death-receptor pathway; mitochondrial pathway; organ dysfunction

Introduction

Sepsis has an incidence of approximately 700,000 cases per year in the United States, and carries an overall mortality rate of nearly 30% [1]. Furthermore, as the American population ages, the incidence of sepsis is projected to increase, as the incidence and mortality rate of sepsis rise steadily with age [1]. The treatment of sepsis, however, utilizing clinical as well as pharmaceutical innovations, has proven to be a difficult task. As human sepsis is a complex and evolving disease, defining both the patient population who may benefit from a potential therapy and the timing of delivery of the therapy is critical. This was evident in the intensive care unit setting, where multiple clinical trials aimed at augmenting hemodynamic parameters of the critically ill patient failed to significantly improve survival [2,3]. The importance of patient selection and timing of therapy was subsequently demonstrated by Rivers et al [4] in an emergency room setting. Patients with severe sepsis or septic shock, defined as having at least two criteria of the systemic inflammatory response syndrome (SIRS) plus a systolic blood pressure no higher than 90mmHg after fluid bolus or a blood lactate greater than four mmol/L, were promptly identified. These patients were then randomized to standard care or an

algorithm aimed at balancing oxygen delivery with demand within six hours. Institution of this algorithm in this defined patient population at this early time point, presumably prior to the onset of irreversible tissue ischemia, significantly improved multiple indicators of organ dysfunction as well as 30-day mortality.

Development of drug therapies to impact the substantial morbidity and mortality of sepsis has been complicated by these same obstacles, with many anti-inflammatory and anti-coagulant drugs showing promise in the laboratory setting, yet no survival benefit in recent randomized human trials [5,6]. However, despite this, recombinant human activated protein C [7], low-dose corticosteroids [8], and intensive insulin therapy [9] have been proven to reduce mortality and have subsequently become widely accepted therapies for treatment of specific populations of septic patients. These clinically successful therapies reduce mortality in part through modulation of the systemic inflammatory response; however, as these drugs (with the exception of corticosteroids) are not classic anti-inflammatory agents, their mechanism of salutary benefit remains to be clarified. Thus, the extent of this interaction as well as the effects on the apoptotic pathway within the immune system is the topic of current investigation. Prior to discussing the apoptotic pathway as a potential target for drug therapy, we will briefly review the current research into the mechanism of the above therapies.

Van den Berghe et al.[9] demonstrated that maintaining the blood glucose between 80 and 110 mg/dL by intensive insulin therapy correlated with an absolute decrease in mortality from 8 to 4.6%, a relative adjusted reduction of 32%, in a surgical ICU population. The greatest reduction of mortality was within the group of patients with multi-organ system failure and a proven septic focus. Intensive insulin therapy also correlated with reductions in the rate of septicemia, time spent on the mechanical ventilator, need for renal replacement therapy, and incidence of critical illness poly-neuropathy. In addition to glycemic control, insulin is essential to serum lipid metabolism; thus, changes in the serum lipid profile may also be involved in the improved outcomes seen in the intensive insulin therapy group. Looking specifically at those patients in the above study who had an ICU stay of greater than seven days, intensive insulin therapy was also found to effect the serum lipid profile. Critical illness was associated with a rise in serum triglyceride levels and a drop in serum high-density lipoprotein (HDL) and low-density lipoprotein (LDL). These changes were significantly reduced by intensive insulin therapy, and this reduction correlated with improved survival [10].

Through modulation of glucose (and potentially free fatty acid) levels, insulin has myriad effects upon the immune response. Hyperglycemia has been shown to alter the immune response in multiple ways, and the administration of insulin has consistently been shown to counteract these effects. For example, hyperglycemia induces expression of leukocyte adhesion molecules, such as intercellular cell adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM), which is suppressed by insulin treatment. Another example is the hyperglycemia-induced impairment of neutrophil function, including chemotaxis, phagocytosis, and respiratory burst, which is attenuated by insulin. Insulin also appears to suppress the release of pro-inflammatory cytokines, which is exacerbated by hyperglycemia [11]. The effect of insulin-induced reduction in free fatty acid (FFA) levels upon the immune response is less clear.

The effects upon the immune response of hyperglycemia and insulin treatment during inflammatory insult are a topic of current experimental interest. In a recent human trial utilizing an intravenous bolus of lipopolysaccharide (LPS) during normoglycemia, hyperglycemic clamp at 15mM (with a subsequent rise in endogenous insulin levels), and hyperinsulinemic euglycemic clamp (HEC), the effects upon TNF- α , interleukin-6 (IL-6), free fatty acid levels, and leukocyte count were analyzed [12]. This model showed no effect in either treatment group upon the TNF- α level; however, the IL-6 level rose significantly higher in both treatment groups

over controls at later time points. In both treatment groups, clamping alone significantly lowered the blood lymphocyte count prior to LPS injection, which persisted throughout the trial, although the blood lymphocyte count declined in all groups. Interestingly, FFA levels were suppressed in both treatment groups. Similarly, in a porcine model, the effects LPS and HEC were recently examined. HEC-LPS significantly lowered TNF- α and glucagon levels as compared to LPS alone. There was no significant effect on interleukin-10 (IL-10), IL-6, or interleukin-8 (IL-8) levels, but again FFA levels were suppressed in both the HEC alone and HEC-LPS groups [13].

The above findings implicate an anti-inflammatory mechanism of intensive insulin therapy, possibly mediated by the reduction of TNF- α or free fatty acid levels. Importantly, insulin by signaling through insulin growth factor receptor also has marked effects on proliferation as opposed to cell death. These seem to be orchestrated by insulin's capacity to activate/phosphorylate the survival factor Akt/PKB [14]. Potential significance of this link has recently been illustrated by Bommhardt et al. [15] who found that overexpression of Akt had a protective effect against lethal polymicrobial septic challenge. Thus far, however, a clear link has not been established between intensive insulin therapy and the apoptotic process. Interestingly, initial studies using the LPS-HEC porcine model have observed that at least with respect to LPS induced apoptosis of T and B lymphocytes, contrary to their hypothesis, HEC alone increased both T and B lymphocyte apoptosis, and the addition of HEC to LPS augmented apoptosis [16]. These studies were limited in that they utilized a relatively short LPS infusion time in an animal model, which does not duplicate the clinical course of the septic patient; however, they do indicate that insulin therapy and normoglycemia effect the inflammatory response and lymphocyte apoptosis. The role of timing of therapy and the extent of this effect upon lymphoid apoptosis in the setting of the septic patient are currently unknown.

The known anti-inflammatory effects of corticosteroids lead to multiple trials utilizing high-dose corticosteroids in septic shock, with variable results. In a meta-analysis of the randomized controlled prospective trials that evaluated the effects of high-dose corticosteroids on mortality in the septic patient, no improvement in mortality was identified [6,17,18]. Recently, with the elucidation that severe sepsis can inhibit the hypothalamic-pituitary-adrenal axis, a renewed interest in corticosteroids has emerged. In a population of catecholamine-dependant patients who fail to respond to a corticotropin stimulation test, a seven-day course of low-dose hydrocortisone and fludrocortisone improved 28-day mortality [8]. A recent metaanalysis including five randomized controlled trials since 1997 confirmed the benefit of low-dose corticosteroid use in vasopressor-dependent septic shock [18].

The beneficial effects of low-dose steroids in vasopressor-dependent patients in septic shock is likely mediated by attenuation of the systemic inflammatory response as well as direct effects on vasomotor tone and an increased responsiveness to vasopressors [19]. The anti-inflammatory effects of corticosteroids have been when established, the full extent of which is beyond the scope of this review. Briefly, the binding of glucocorticoids to glucocorticoid receptor and interactions with glucocorticoid responsive elements within the nucleus can block production of many inflammatory cytokines in multiple cell types. Glucocorticoids also block the production of inflammatory mediators, such as cyclo-oxygenase-2, and decrease leukocyte adhesion to endothelium [19].

In regards to apoptosis, corticosteroids/glucocorticoids have also shown both potentiating and suppressive effects on the process of apoptosis, which appears to be cell/tissue specific in nature. In thymocytes, proliferating lymphocytes, and mast cells, corticosteroids largely produce pro-apoptotic effects[20,21], however, in neutrophils, epithelial cells and fibroblasts, the effect is typically anti-apoptotic [22,23]. With respect to sepsis, the experimental data, thus far, indicate that corticosteroid release during sepsis appears to increase apoptosis of

thymocytes [24], which is mediated by induction of caspase-9 [25]. However, the effect of low-dose steroids in the experimental setting of either animal sepsis or in vasopressor-dependent septic shock on the onset of apoptosis in immune or non-immune tissue remains to be understood. The concept of modulating the systemic inflammatory response to severe sepsis lead to many anti-inflammatory agents reaching randomized controlled clinical trials. Anti-endotoxin, anti-CD14, anti-LBP, anti-platelet activating factor, anti-TNF, and anti-IL-1 therapies went to phase III trial, and all met with limited success, showing no effect on mortality [5,6]. Derangements of the coagulation cascade in severe sepsis have also lead to multiple trials of anti-coagulant drugs within the last decade. Large randomized controlled trials of anti-thrombin-III and Tissue Factor Pathway Inhibitor showed no improvement in survival [5,6].

Recombinant human activated Protein C (rhaPC), on the other hand, was found to reduce 28-day mortality by 6%, a relative reduction of 19%, in patients with a documented or suspected infectious source, signs of the systemic inflammatory response syndrome, and evidence of organ dysfunction [7]. D-dimer as well as IL-6 levels were significantly reduced during rhaPC infusion, evidence of the anti-thrombotic and anti-inflammatory effects. A subgroup analysis of this study showed a decreased effectiveness with lower APACHE II scores at admission, prompting the Food and Drug Administration to approve the drug for use in patients with severe sepsis with high predicted mortality [26]. This finding was recently confirmed in a follow-up study involving patients with severe sepsis and a low risk of death, defined as an APACHE II score less than 25 or single-organ failure. The study was halted at interim analysis due to low likelihood of demonstrating a benefit of treatment and a higher risk of serious bleeding in the rhaPC group [27].

The successful application of rhaPC and the concurrent failure of other anticoagulant trials lead to investigation into the mechanism of action of rhaPC. The coagulation cascade is intimately tied to the mechanisms of the inflammatory response, and aPC exhibits effects on both, thus inhibiting the inflammatory response through both indirect and direct mechanisms. Inhibition of thrombin formation directly and via inactivation of factor Va and VIIIa attenuates thrombin-induced inflammatory cytokine release and endothelial dysfunction [28].

Recombinant human activated Protein C also directly attenuates the inflammatory response of human endothelial cells via binding to protease-activated receptor-1 (PAR-1) in an endothelial protein C receptor (EPCR) dependant manner [29]. Joyce et al. [30] demonstrated that rhaPC alone down-regulated the expression of nuclear factor-kappaB (NF- κ B) and attenuated NF- κ B expression following TNF- α induction. Furthermore, TNF- α induced expression of NF- κ B regulated adhesion molecules, such as ICAM, VCAM, and E-selectin, was inhibited by rhaPC, potentially inhibiting leukocyte adherence *in vivo*.

The effect of rhaPC on microcirculation *in vivo* was recently demonstrated in a rat model utilizing intravital microscopy following LPS versus LPS plus rhaPC injection [31]. At one, two, and three hours post-injection of LPS alone or LPS plus either low or high-dose rhaPC, the mesenteric microcirculation was examined under intravital microscopy. Both treatment groups had a significantly lower number of adherent leukocytes per field than the control group. The low-dose rhaPC group was found to have a significantly lower number of micro-vascular bleeding events and a higher arteriole and venule red blood cell velocity than both the control and high-dose rhaPC group. In addition to the effects upon microcirculation, the treatment groups were found to have an attenuation of the drop in white blood cell and platelet count that was evident in the LPS treated group. In addition, LPS also induced an early rise in TNF and later rise in IL-6, both of which were attenuated by rhaPC treatment. A possible correlation with ensuing organ damage was also demonstrated by rise in alanine transaminase and blood urea nitrogen in the LPS group, which was blocked by rhaPC treatment.

Evidence for alteration of leukocyte migration is also shown in a recent human study in which the accumulation of leukocytes, predominantly neutrophils, in the lungs following intra-bronchial LPS instillation was inhibited in patients treated with rhaPC; furthermore, these neutrophils exhibited decreased *ex-vivo* chemotaxis [32].

Activated protein C has similarly been shown to counter the induction of apoptosis in animal studies as well as in human endothelial and monocyte cell lines *in vitro* [28,30,33,34]. Recombinant human activated Protein C prevents staurosporine-induced apoptosis in human endothelial cells and up-regulated expression of multiple anti-apoptotic genes, including endothelial nitric oxide synthase (eNOS), AI, Bcl-2 homologue, and inhibitor of apoptosis-1 [30]. Apoptosis in a human monocyte cell line is also inhibited by rhaPC, an effect that is mediated by EPCR [28]. In human brain endothelial cells subjected to hypoxic injury, Cheng et al. [33] found a 60% reduction in apoptosis with the addition of rhaPC, and demonstrated that this cytoprotective effect depends upon EPCR and PAR-1 binding of rhaPC. In addition, hypoxia increased levels of the pro-apoptotic molecules p53, Bax, and caspase-3 and decreased Bcl-2 levels, an inhibitor of apoptosis. All of these changes were attenuated by the addition of rhaPC.

In an *in vivo* murine model of ischemic stroke, the application of rhaPC decreased the size of infarction and edema formation, and decreased neutrophil infiltration and endothelial ICAM-1 expression [35]. Cheng et al. [33], in a similar model, demonstrated that the rescue effect of rhaPC is mediated by EPCR and PAR-1. A direct neuroprotective effect of aPC was recently shown *in vitro* and *in vivo* following N-methyl-D-aspartate (NMDA) and staurosporine induction of apoptosis [36]. In NMDA induced apoptosis, aPC was found to decrease translocation of apoptosis-inducing factor (AIF) into the nucleus, block caspase-3 and p53 induction, and attenuate the increase in Bax and decrease in Bcl-2. Activated protein C also blocked caspase-8 activation, thus blocking staurosporine-induced apoptosis. In both NMDA and staurosporine-induced apoptosis, the effects of aPC were dependant upon PAR-1 and PAR-3.

These insights into the mechanisms of the current clinically effective drug therapies for severe sepsis indicate that the apoptotic pathway itself may prove to be a target for future drug therapies.

Apoptotic Pathways

The apoptotic process is one in which cells are actively eliminated via a programmed pathway during morphogenesis, tissue remodeling, and the resolution of the immune response. Inducers of apoptosis include steroids, cytokines such as TNF- α , IL-1 and IL-6, FasL, heat shock, oxygen free radicals, nitric oxide and FasL-expressing cytotoxic T lymphocytes (CTLs) [37]. 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 or stress-induced pathway (**Figure 1**). In type I cells, Fas antigen (CD95), a major death receptor that belongs to the TNF superfamily of membrane receptors, is the first component of the pathway to receive a death signal. 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 non-immune cells in the liver, lung and heart [38]. When Fas binds to its ligand, FasL, it trimerizes and creates a death-induced signaling complex (DISC) which recruits an adaptor molecule also containing a death domain, known as Fas-associated death domain (FADD). FADD binds to these activated death domains and to pro-caspase 8 through death effector domains (DEDs) to form the DISC. The death signal is then transduced from the DISC to a downstream caspase cascade when pro-caspase 8 is cleaved and becomes active caspase 8, which can, in turn, cleave and activate downstream effector

caspases, such as caspase 3, 6, or 7. Caspase 3 cleaves inhibitors of caspase activated DNase (ICAD) and cleaves DNA in the nucleus [39], which leads to apoptosis.

In type II cells, almost no DISC is formed, and the mitochondria is essential for releasing cellular destruction molecules such as cytochrome c which activates downstream caspases such as caspase 3 and caspase 9 (**Figure 1**). The initiation of this pathway, however, is not well defined. The pathway can be activated by loss of growth factors such as IL-2, IL-4, or GM-CSF, the addition of cytokines such as IL-1 and IL-6, or exogenous stressors such as steroids, reactive oxygen intermediates, peroxynitrite or nitric oxide (NO) which in turn activate pro-apoptotic members of the Bcl-2 family. Pro-apoptotic 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 mitochondrial membrane potential ($\Delta\psi_m$). Anti-apoptotic members of the Bcl-2 family (Bcl-2, Bcl-xL) block the release of cytochrome c, Smac/Diablo, and apaf-1 from the mitochondria, which, via apoptosome formation can activate caspase 9, which can in turn activate downstream caspase 3. Since apoptosis in type II cells can depend on the balance of the Bcl-2 family members, a dominance of anti-apoptotic family members such as Bcl-2 and Bcl-xL can promote survival of the cell [40]. The endoplasmic reticulum/stress-induced pathway is the least understood of the apoptotic pathways and appears to involve the activation of caspase 12 by Ca^{2+} and oxidant stress [41] (**Figure 1**) [42].

The Role of Apoptosis in the Pathology of Sepsis

As we have already alluded to in the introduction, advances have been made in the treatment of the septic patient via the application of APC, low-dose steroid therapy and insulin to lock blood glucose levels. These are still somewhat modest effects, however, which are evident on select patient groups. While these agents all appear to commonly affect inflammation, the failure of more directed anti-inflammatory therapeutics as well as important non-inflammatory targets of these treatments, suggests that other aspects of the pathology of sepsis may be targets. In this regard, studies in recent years have suggested that dysregulated apoptotic immune cell death may play a role in contributing to the immune dysfunction and multiple organ failure observed during sepsis and that blocking it can improve survival of experimental animals [43-46]. The immune cells most affected by this dysregulated apoptotic cell death appear to be lymphocytes. This loss of lymphocytes is detrimental to the survival of septic mice, as we also know that RAG1-/- mice have a markedly decreased ability to survive cecal ligation and puncture (CLP) as compared to their wild type counterparts [47]. Apoptosis of lymphocytes is frequently seen 12+ hours following the onset of experimental polymicrobial sepsis in the thymus, spleen, and gut-associated lymphoid tissues (GALT). It has been suggested that dysregulated lymphocyte apoptosis in these experimental animals results in decreased septic survival through the loss of lymphocytes. This, in turn is speculated to lead to immune suppression leaving the mouse unable to fight the lethal effects of sepsis, resulting in multiple organ failure and eventual death. Lymphocyte apoptosis in the thymus appears to occur early after the onset of sepsis (4 hours) and is independent of the effects of endotoxin or death receptors [48], but appear to be the result of glucocorticoids and NO [24]. It is also thought that the early release of complement C5a may contribute to thymocyte apoptosis [49]. In the bone marrow and lamina propria B cells [50], splenic T cells, intestinal intraepithelial lymphocytes (IELs), and mucosal T and B cells of the Peyer's patches [51], apoptosis is mainly death receptor-driven. Apoptosis in the spleen particularly seems to be important in septic mortality as an increase in splenic lymphocyte apoptosis in experimental animals after CLP results in reduced survival [52]. Lymphocyte restricted overexpression of Bcl-2, however, ameliorates this condition, further supporting the concept of lymphocyte loss as being an important aspect of increased mortality in experimental sepsis [47].

Other immune cell types, which have been reported to exhibit an increased incidence of apoptosis in sepsis, are CD8⁺ lymphoid-derived dendritic cells of the spleen after CD3⁺CD4⁺ T cell activation [47], but the significance of this change is not well understood. Certain non-immune cells have also been shown to exhibit apoptotic changes, including mucosal epithelial cells [53] and to a certain extent endothelial cells [54,55]. As mentioned earlier discussion of APC these latter non-immune cell targets, like the endothelium, may important targets of the pathological effects of apoptosis.

Apoptotic Targets and Therapeutic Considerations

Targeting the intrinsic and extrinsic pathways

One of the earliest anti-apoptotic approaches in sepsis research was the attempt to inhibit caspase activation. Caspase inhibitors usually contain fluoromethyl ketones (fmk) or chloromethyl ketones (cmk) that are derivatives of peptides that imitate cleavage sites of known caspase substrates. To inhibit the activity, they irreversibly alkylate the cysteine residue on the active site of the caspase [56]. Caspase-specific inhibitors that have been used include z-DEVD-fmk (caspase 3 and 7) and Ac-YVAD-cmk (caspase 1) [57] (**Table 1**). It has also been shown that broad spectrum caspase inhibitors such as z-VAD-fmk can prevent lymphocyte apoptosis in sepsis, and in turn, improve septic animal survival by 40-45% [58,59]. However, at high doses, caspase inhibitors can have non-specific effects and cause cytotoxicity. In this respect, a different kind of pan-caspase inhibitor called Q-VD-Oph has been studied, however, that potently inhibits apoptosis but is not toxic at high doses, unlike z-VAD-fmk or Boc-D-fmk. Since it is also equally effective at preventing apoptosis by the three major apoptotic pathways (i.e. caspases 9/3, caspases 8/10, and caspase12) it seems likely that the efficacy of caspase inhibitors may be enhanced by using carboxyterminal o-phenoxy groups to make them more efficient in the clinical setting [60].

Peptidomimetics represent another potentially useful method of targeting the apoptotic pathway. "Peptidomimetics" are mimics that have similar structure and functional properties of the native parenteral peptides. This approach was adopted since the use of biologically active peptides as pharmaceutical compound have more or less failed due to their inability to stay bioavailable, penetrate cell membranes, and maintain metabolic stability. Synthetic mimics, however, can be generated to be more conformationally stable compounds that resist enzyme degradation, can cross cell membranes, and target specific proteins [61]. This new type of approach is being studied at present for its potential pro-apoptotic effect, particularly in cancer. Here they are being used to mimic protein-protein interactions that will activate the apoptotic pathway in tumors, and in doing so, kill apoptosis-resistant tumors. Mimics have been synthesized that resemble the death domain of the pro-apoptotic Bcl-family member Bid [62] and also Smac, the pro-apoptotic protein which acts as an antagonist of the inhibitor-of-apoptosis (IAP), which normally suppresses caspases [63]. Both of these peptidomimetics permitted caspase activation and induced apoptosis in human cancer cells. The methods used to generate these mimics, hydrocarbon stapling [62] and the use of nonnatural amino acid replacements[63], resulted in protease-resistant molecules that were able to cross the cell membrane and bind efficiently to their target proteins. Since the problem with most peptide-based therapies is proteolytic degradation, the conformation of a peptidomimetic is not favorable for this [64]. Peptidomimetics are also water-soluble and are non-immunogenic. This allows them to be administered for longer periods of time [65]. It, therefore, seems possible to speculate that peptidomimetics may be useful in future therapies that need to modulate protein-protein interactions, and in the case of sepsis, activate anti-apoptotic proteins, which may protect those cells from apoptosis normally lost during the course of the disease. This idea may also be an alternative to gene therapy, which, in the past, has not done well in the clinical setting [66].

Enhancement of anti-apoptotic proteins, such as Bcl-2, has been shown to produce almost complete protection against T cell apoptosis in transgenic mice that overexpress Bcl-2. This, in turn, improved their survival after sepsis [47,59]. In addition, adoptive transfer of T cells from Bcl-2 overexpressing mice into wild type septic mice also improved their survival [58]. While this clearly illustrates the important role of the lymphocyte in sepsis in controlling infection, it also illustrates a clear therapeutic target, which can be used to restore lymphocytes lost during this state.

Yet another target that decreases lymphocyte apoptosis is Akt, a regulator of cell proliferation and death. It has been shown in mice that overexpress Akt that lymphocyte apoptosis is decreased and survival after CLP is improved to 94% [15]. It may be at this level, (i.e. the activation of Akt) that treatments such as glucose control with insulin therapy or low-dose steroids have an effect on apoptosis in septic individuals. In addition, IFN- γ (a potent macrophage activator) release capacity is improved with Akt overexpression. The presence of IFN- γ is thought to be therapeutic itself, as restoration of macrophage function has been shown to alleviate sepsis in humans that showed monocyte deactivation and loss of Th1 cytokines [67].

Based on the finding of increased Fas expression in the tissues of septic mice [44,45,68,69], there has been increasing interest in targeting components of the death receptor/extrinsic pathway in an attempt to ameliorate the effects of conditions affected by dysregulated apoptosis, such as sepsis. In this regard, studies from our own lab had initially focused on blocking the pathway at the death receptor itself (Fas). Initially, animals were treated with Fas fusion protein (FasFP; Amgen Inc., Thousand Oaks, CA) to inhibit the receptor ligation. This treatment resulted in a survival benefit [44] and reduced hepatic injury while improving total hepatic, intestinal and cardiac blood flow during sepsis [45]. In these studies it was also observed that when FasFP was given at 12 hours post-CLP but not earlier (0 hour) it was shown to have a positive effect [44]. This observation suggests that pathological events such as the septic aberrations in organ damage and/or blood flow which develop late in the course of sepsis [70], remain amenable to delayed FasFP treatment. In addition, a cell survival tyrosine kinase (MET) has been found to sequester Fas on hepatocytes but inhibiting Fas self-aggregation and Fas ligand binding [71]. However, limitations such as short half-life of these large fusion protein and/or antibody constructs as well as issues of tissue specific bio-availability represent important limitations of this type of intact protein treatment. One solution would be a possible formulation of peptidomimetics, as mentioned previously, that can block Fas.

More recently, interfering RNA technology has been utilized to target gene expression of members of the extrinsic death receptor/Fas pathway. The experience with double stranded small interfering RNA (siRNA) (against Fas and caspase 8) has proven somewhat different however. This is most likely related to the biology of siRNA and its function. Both Fas and caspase 8 siRNA have been used in models of fulminant hepatitis [72,73], and most recently, in sepsis [69]. Fas siRNA given 30 min. after CLP improved survival by 50% while reducing indices of organ damage and apoptosis in both the liver and spleen [69]. The mechanism of this survival benefit is still, for the most part, unknown. Preliminary data from our lab suggest, however, that Fas siRNA can be taken up by CD4⁺ and CD8⁺ T cells, as well as B cells. In the case of the spleen, it appears that lymphocyte apoptosis is reduced by silencing Fas, therefore enabling the host to maintain innate and adaptive immune cell crosstalk that would aid it in developing an immune response to ward off the infectious challenge. With respect to the liver, preliminary results suggest that Fas siRNA reduces the recruitment of potentially tissue damaging lymphocytes. It has been suggested in a model of hepatitis C that Fas ligand expressing CD4⁺ T cells can induce chronic hepatic inflammation [74], which, in the case of sepsis, may be instrumental in initiating multiple organ failure. By the same token, experimental CLP mice lacking CD8⁺ T cells exhibit improved survival over wild type [75].

It remains to be established what the exact link between increased Fas expression and the recruitment of potentially cytotoxic pro-inflammatory cells in the liver might be that leads to the development of organ damage/failure and eventual death.

As intriguing as this approach is, several hurdles need to be overcome beyond cell targeting before siRNA can be applied clinically. Being double stranded RNA, the question can be raised as to whether siRNA is able to induce IFN signaling. Such signaling might induce unwanted pro-inflammatory sequelae in the septic animal undergoing siRNA treatment. While this is still under investigation, there are studies primarily in mammalian cell lines, which suggest that siRNA can induce interferon under some conditions [76,77]. On the other hand, we found that the hydrodynamic injection of naked siRNAs (50 µg/mouse) in mice was not capable of inducing IFN- α or IL-6 [69]. This is in keeping with another recent *in vivo* study, which has suggested that naked siRNA (as was delivered in our study) is not able to induce IFN through TLR3, unlike poly(I:C) [78]. Probably the biggest hurdle facing the use of siRNA clinically is that of its delivery. While a hydrodynamic-based method (where volume and rate of injection are critical in the uptake of naked constructs) is easily employed in research [79], it clearly cannot be used in human therapy in this way. Because naked siRNAs are degraded within seconds from a low volume injection, there needs to be a carrier or delivery vehicle that will protect the siRNA while not requiring a high volume, rapid injection. Cationic liposomes encoding anti-TNF siRNA have been studied, and appeared to reduce TNF- α levels after endotoxemia, and may represent a useful alternative method for encapsulating/delivering siRNA [80,81]. Other investigators have suggested the use of vectors as another mode of targeted siRNA delivery. However, as vectorized gene delivery these may cause inflammation themselves [76]. With respect to the extent of silencing, we and others have found that hydrodynamic injection of siRNA is able to maintain suppression of its target mRNA, at least in the liver, for up to 10 days following injection, with the signal starting to return at day 14 [69,72]. With the major target of this hydrodynamic form of injection being the liver, we can only speculate that since the cells in the liver do not divide like cell lines in culture, there is less dilution of functional siRNAs which may allow for the sustained suppression of mRNA signal for a longer period of time [72]. Hypothetically, since this is a prolonged silencing effect and not a permanent one, toxicity would not be of high concern.

Other agents that affect apoptosis

There have been other agents reported that are not direct inhibitors of programmed cell death *per se*, but have been discovered to affect the apoptotic pathway indirectly. In this regard, there is strong evidence for complement activation following CLP and in human sepsis [82], and that elevated levels of the anaphylatoxins C3a and C5a are thought to contribute to early thymocyte apoptosis. It was found that blockade of C5a by i.v. injection of rabbit anti-rat C5a antibody in septic rats reduced thymic apoptosis, completely inhibited the activation of caspases 3, 6, and 9 and restored expression of Bcl-xL [49]. Since C5a is not a direct member of the apoptotic pathway, it is thought that since complement activation promotes the release of pro-inflammatory cytokines such as TNF- α , IL-1 β , IL-6, and IL-8 from leukocytes [83], that it may affect the balance of cytokines that promote apoptosis but without affecting NF- κ B. Thus, this could be a reasonable explanation as it has been seen that thymocyte apoptosis in sepsis is independent of endotoxin or TNF receptors such as Fas [24,84]. Lastly, blocking C5a may also reduce neutrophil chemotaxis that may prevent excessive organ damage in this fashion [85].

Other agents have been tried that may reduce apoptosis after sepsis *in vivo*, as they are known to inhibit apoptosis *in vitro*. The protease inhibitor class of anti-retroviral agents were introduced in CLP mice as a pre-treatment and post-treatment and found to increase survival in both cases. This therapy reduced lymphocyte apoptosis, early TNF- α levels and late IL-6

and IL-10 levels. Because the treatment had no effect on RAG1^{-/-} mice, this suggests that it is specific for lymphocyte apoptosis [86]. However, like anti-C5a, it may also inhibit neutrophil influx into septic animal tissues/organs [87].

Another strategy showing promise includes the use of the vasodilator adrenomedullin and adrenomedullin binding protein (AM/AMBP1), which have been shown to increase survival of septic mice by 50% by preventing sepsis-induced vascular endothelial cell apoptosis. Treatment of mice with AM/AMBP1 increased protein levels of anti-apoptotic Bcl-2, while limiting tissue injury, improving organ blood flow and preventing the progression of sepsis from the hyperdynamic phase to the hypodynamic phase [55]. Interestingly, while the effect of lymphoid cell apoptosis remains to be established, its effects on endothelial cell apoptosis are in keeping with one of the suggested anti-apoptotic targets of APC [30].

Conclusions

Despite overwhelming research efforts and clinical trials, there has yet to be a therapy offered that significantly modifies the outcome of this disease. Even though there have been promising candidates for therapeutic intervention, sepsis manifests itself as multiple processes, making this task difficult. Here we have considered how recent treatment advances, such as APC administration, low-dose steroids and the application of insulin to control the septic patient's blood glucose, levels may therapeutically speak through action on the apoptotic process. We have also reviewed several experimental studies focusing on the apoptotic arm of sepsis, revealing several targets (**Table 2**) within the apoptotic pathway, which may be useful in designing stand-alone and/or adjuvant therapies that may have a greater impact on septic mortality. Together these findings will hopefully reveal novel therapeutic targets and approaches that can improve the survival of the critically ill septic patient.

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Figure 1.

Both the extrinsic and intrinsic arms of the death pathway contain possible targets that can be exploited for therapy.

Table 1

Caspase Inhibitors

Caspase	Inhibitor
1	Ac-YVAD-cmk [57]
2	z-VDVAD-fmk
3	z-DEVD-fmk
4	z-LEVD-fmk
5	z-WEHD-fmk
6	z-VEID-fmk
7	z-DEVD-fmk
8	z-IETD-fmk
9	z-LEHD-fmk
10	z-AEVD-fmk
12	z-ATAD-fmk
13	z-LEED-fmk
family	z-VAD-fmk [58,59]
family	Boc-D-fmk
3,8,9,10,12	Q-VD-Oph [60]

Table 2
Potential Therapeutic Targets in the Apoptotic Pathway

Target	Potential therapies
Fas death receptor	FasFP [44], Fas siRNA [69]
Caspases	Inhibitors- broad and specific [58,59], siRNA [69]
Bcl-2 family	Overexpression of anti-apoptotic members (gene therapy) [47,59] Adrenomedullin/AMBP1 [88], Possible use of peptidomimetics [61]
Proteases	Protease inhibitors [88]
Akt	Overexpression of (possible gene therapy) [15]
C5a	anti-C5a neutralizing antibody [49]