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NF-κB ACTION IN SEPSIS: THE INNATE IMMUNE SYSTEM AND THE HEART

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1. ABSTRACT

Sepsis is the clinical syndrome that results from a host's inflammatory response to infection via activation of the innate immune system. This response involves a complex network of inflammatory mediators that is self-reinforcing. When this immune response progresses uncontrollably, it can ultimately result in cardiovascular collapse and death. This complex inflammatory response is comprised of multiple mediators including cytokines such as TNF-α and IL-1β, that are synthesized and secreted in response to signaling by receptors of the Toll-like receptor (TLR) family of pattern recognition receptors (PRR) that bind to pathogen associated molecules. A central downstream element of TLR-dependent signaling is the pleiotropic transcription factor NF-κB. NF-κB has been implicated in the regulation of multiple biological phenomena and disease states, including apoptosis, cell growth, stress response, innate immunity and septic shock. NF-κB-dependent genes are numerous and several have been implicated in the pathogenesis of sepsis and associated with cardiac dysfunction in sepsis. NF-κB activation occurs in multiple organs and cell types, and may be primarily protective in one tissue but injurious in another. Thus, a detailed understanding of the molecular basis of the pathophysiology of sepsis is needed in order to specifically block pro-inflammatory and pro-apoptotic signaling in the heart, while avoiding adverse effects in other organs.

2. INTRODUCTION

Sepsis is a serious clinical problem with an incidence of 400,000-750,000 per year and is lethal in 20-30% of patients (1-6). Sepsis is an inflammatory response to infection that is primarily elicited by activation of the innate immune system, which induces the synthesis and release of inflammatory mediators, including the cytokines TNF-α and IL-1β from monocytes and other cells (Figure 1). These cytokines stimulate polymorphonuclear leukocytes and endothelial cells to release downstream inflammatory mediators, including platelet activating factor, nitric oxide (NO) and prostaglandins, as well as anti-inflammatory mediators such as IL-10, transforming growth factor beta (TGF-β) and IL-1R antagonists (6-11). It is likely that the relative activation of pro- and anti-inflammatory mediators determines, in part, the severity of the septic reaction. In instances where the inflammatory reaction is severe, homeostasis of the cardiovascular system is affected (Figure 2) and septic shock, characterized by myocardial depression and hypotension, follows. The majority of fatalities from sepsis occur consequent to hypotension that is refractory to volume resuscitation and to cardiovascular collapse.

The activation of cytokines and their receptors mediates a large part of the systemic response to sepsis. These signaling pathways are activated by pattern recognition receptors (PRR) of the Toll-like family of receptors (TLRs) that bind to molecules synthesized or released by pathogenic organisms. A central downstream element of many of theses TLR-dependent signaling pathways is nuclear factor kappaB (NF-κB), which triggers expression of genes encoding inflammatory cytokines and other inflammatory mediators. NF-κB is a pleiotropic transcription factor implicated in the regulation of multiple biological phenomena and disease states, including B-cell development, innate immunity, activation of acquired immunity, pattern formation, inflammation, cell growth and death, stress and ischemic responses, cachexia, atherosclerosis, asthma, arthritis, diabetes, stroke, sepsis and septic shock. NF-κB was first discovered in 1986 as a B-cell specific DNA binding protein that binds the kappa light chain enhancer region (12). To date, five mammalian NF-κB subunit genes have been characterized, Rel A (p65), Rel B, NF-κB1 (p50/p100), NF-κB2 (p52/p105) and c-rel, in addition to the protooncogene v-rel. All Rel-family proteins contain both a Rel-homology domain (RHD), a

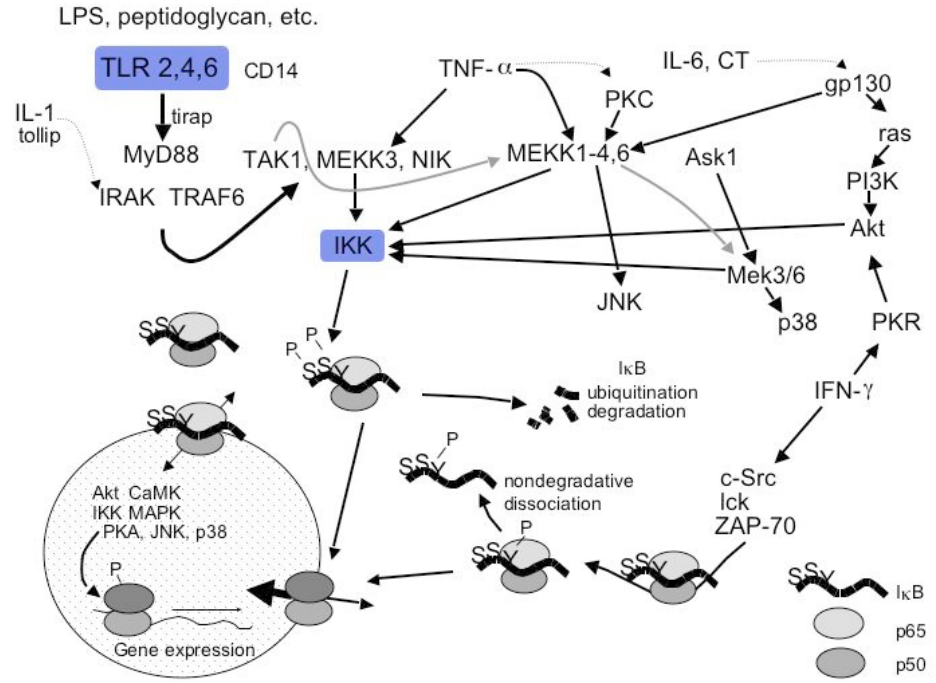

Figure 1. NF-κB signaling network in sepsis. NF-κB is activated primarily through IκB phosphorylation which is mediated by multiple signal transduction cascades, including TLR-, cytokine- and PKC-mediated signaling through MEKK, Tak1 and NIK. Ultimately, serine phosphorylation of IκB occurs via activation of the IKK complex which is an integration point for cytokine and MAPK signaling cascades. Tyrosine phosphorylation is known to be mediated in part by c-Src. Additionally, multiple signaling proteins, including MAPKs and Akt, are capable of phosphorylating NF-κB subunits directly, which plays a major role in modulating the transactivational activity of NF-κB at the promoter. Thus, NF-κB is positioned as an integrator of multiple diverse parallel signaling cascades.

300 amino acid length domain that functions in DNA binding, dimerization and interaction with the inhibitory κB (IκB) proteins, and a nuclear localization domain. In addition, members of one subgroup, including p65, Rel B, c-rel and v-rel, contain one or more transcriptional activation domains. Members of the second subgroup, NF-κB1 and NF-κB2, lack transcriptional activation domains and contain large carboxy-terminal domains that are highly homologous to the natural inhibitor of NF-κB (IκB) protein. Thus, the NF-κB1 and NF-κB2 proteins contain their own repressors in their complete forms (p100 and p105, respectively), which are removed during processing to the active p50 and p52 subunits. Functional NF-κB dimers containing multiple subunit combinations are known to exist, the most common being p65/p50, RelB/p50 and c-rel/p50. Different heterodimers are known to have differing affinities for variants of the NF-κB consensus DNA binding site and this may contribute to differential regulation of NF-κB–dependent genes (13, 14). Additionally, NF-κB homodimers are known to exist, particularly p50/p50, which is implicated in repression of gene expression (5).

NF-κB dimers are complexed with inhibitory proteins of the IκB family in the cell. Members of the IκB protein family contain ankyrin repeats that bind to the RHD portions of NF-κB proteins, thereby reducing nuclear localization and DNA binding of NF-κB. To date, there are seven known mammalian IκB proteins, the most common of which are IκBα and IκBβ. Specific stimuli activate NF-κB by inducing the phosphorylation and subsequent degradation or dissociation of IκB proteins from NF-κB, which enhances nuclear localization and DNA binding. Phosphorylation of two closely placed serines in the amino-terminal regulatory domain of IκB proteins (Ser32 and Ser 36 in the case of IκBα) results in site-specific ubiquitinization and proteosome-mediated degradation of IκB (Figure 1). A non-degradative pathway, which has been studied to a much lesser extent, involves tyrosine posphorylation (Tyr42 for IκBα) (15). Although this pathway is known to involve the tyrosine kinases c-Src, p56 and ZAP-70, the terminal kinase is unknown and the upstream activators are ill-defined (15-17). In addition to these known regulatory phosphorylation sites, there are additional Ser, Thr and Tyr sites which are phosphorylated in the carboxy-terminal PEST domains of IκB proteins. Phosphorylation of these sites influences protein stability and half-life and, although in some cases required for IκB degradation, are not sufficient for NF-κB activation (14). The most recent model for NF-κB regulation proposes that IκB proteins act by maintaining an equilibrium in which NF-κB/IκB complexes are predominantly cytoplasmic and by inhibiting NF-κB DNA binding activity (18-21). These and other aspects of NF-κB and IκB protein families and regulation are reviewed in detail by Baldwin (14).

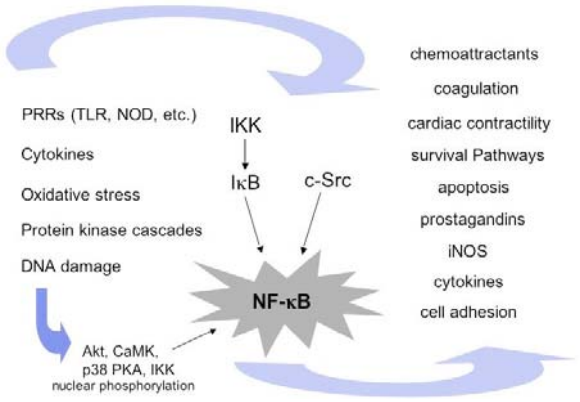

Figure 2. Effects of NF-κB signaling in sepsis. NF-κB activity integrates signals from a diverse set of receptors, proteins and signaling cascades in response to specific stimuli (left) and regulates a wide variety of gene products with affects upon multiple cellular functions that impact cardiovascular physiology and pathophysiology during sepsis (right). The overall effect of NF-κB activation depends upon crosstalk between multiple signaling cascades and the influence of feedback circuits that strengthen or reduce inflammatory signaling. Runaway NF-κB activation reinforces activation of gene programs that are pro-cell death, reduces cardiac function and enhances coagulation and inflammatory reactions that eventuate in cardiac dysfunction and death.

3. SIGNAL TRANSDUCTION AND NF-κB

Although numerous signaling pathways are known to activate NF-κB (Figure 1), the canonical pathway involves the NF-κB-inducing kinase (NIK) and the IκB kinase (IKK) complex and subsequent degradation of IκB proteins. These two kinases, NIK and IKK, serve as signaling "hubs" that connect multiple upstream cascades to NF-κB activation forming a complex signaling network. Many cytokines, including TNF-α and IL-1β, activate NF-κB predominantly via this pathway, TNF-α being the most thoroughly studied of these. TNF-α is the prototypical member of the death receptor superfamily of cytokines and signals through two receptors, TNRF1 (p55) and TNRF2 (p75). TNF-α acts via trimerization of its receptor(s) and the multimerized intracellular domains of the receptors interact via cystein-rich domains (CRD) with members of the TRAF (TNF-receptor associated factor) adapter family. TRAFs, either alone or in combination with the adapter proteins cIAP1/2, RAIDD or RIP, activate NIK and/or MEKKs (i. e. MAP3Ks), which ultimately activate NF-κB and MAPK cascades (Figure 1). It is thought that some of the TRAF proteins and adapters form a complex with NIK, and perhaps the IKK proteins, during the process of IKK activation. The IKK proteins comprise the IKK signalsome, an important signaling complex which is part of the TNF-α cascade, the TLR cascades involved in sepsis (discussed below) and acts as a key hub in integrating MAPK, PKC and PI3K/Akt signaling for NF-κB activation (Figure 1) (22-25). The IKK signalsome consists of two IKKα (IKK1), two IKKβ (IKK2) and two IKKγ (NEMO) subunits. The IKKα and IKKβ kinases specifically catalyze the phosphorylation of Ser32 and Ser 36 of IκBα. Recent evidence suggests the existence of novel IKKs and IKK complexes, although the associated signaling cascades and their significance are yet to be determined (26-29).

Another family of cytokines, the interleukins (ILs), consists of more than 15 members that fall into two classes, the first of which employs adapter proteins called IRAKs, similar to TRAFs, and the second of which is linked to the Jak/Stat pathway via gp130. Both classes of ILs are able to activate NF-κB (Figure 1). IL-1β is the prototypical member of the first class and IL-6 is the prototypical member of the gp130 associated family of interleukins, which also includes cardiotrophin (CT-1), leukocyte inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF). Cytokine expression from immune responsive cells plays an important role in cardiac pathophysiology in response to sepsis. Cardiomyocytes themselves are capable of expressing cytokines, including TNF-α IL-6 and IL-1β as well their receptors, and many of the major actions of cytokines upon the heart are due to their action upon cardiomyocytes (30-32). Cytokines also play important roles in sepsis, and much of this cytokine expression occurs as a result of NF-κB-dependent genes, as will be discussed. NF-κB activation in response to sepsis occurs primarily by signaling through the toll-like family of cellular receptors.

4. TOLL-LIKE RECEPTORS, SEPSIS AND NF-κB

In 1999, Qureshi *et al.* demonstrated that spontaneous mutations associated with hyporesponsiveness to lipopolysaccharide (LPS) are linked to the gene encoding TLR4 (33). Other investigators, using mice bearing a targeted disruption of *TLR4*, demonstrated a direct relationship between LPS response and TLR4 function (34). Subsequent studies have confirmed that TLR4 is the primary receptor for LPS from gram-negative organisms, bacterial lipoproteins and yeasts, while TLR2 is the PRR for gram-positive organisms (35-38). Regardless of the receptor, the activation of NF-κB by bacterial products during sepsis leads to production of large amounts of proinflammatory molecules, which results in tissue damage and organ failure characteristic of sepsis and can result in death. In fact, high levels of NF-κB activation in peripheral blood mononuclear cells are associated with a significantly higher mortality in human sepsis and was a better predictor of outcome than physiologically based scores (39, 40).

TLRs have a single-pass transmembrane domain and an extracellular domain containing leucine-rich repeats. The intracellular toll/IL-1R/resistance (TIR) domain is shared by receptors of the TLR and IL-1R families, however, the IL-1R family receptors have a different extracellular region consisting of immunoglobulin domains (41, 42). The prototypical member of the TLR family, the *Drosophila* Toll receptor, is involved in developmental processes and in innate immunity against fungal pathogens (43-45). Interestingly, although the *Drosophila* Toll receptors respond to fungal and gram positive bacterial infections, the response to gram negative infection is via a separate receptor, yet to be identified. This unidentified receptor, which responds to LPS, acts via the adapter

protein IMD, which appears to be a RIP analogue (46). Nevertheless, both *Drosophila* receptor types activate signal transduction cascades that ultimately involve different members of the *Drospophila* NF-κB family resulting in activation of different subsets of antibacterial and antifungal genes. Interestingly, although all known mammalian TLRs, of which there are at least ten, act via the same signal transduction cascades, different but overlapping sets of downstream genes are activated. As mentioned previously, TLR4 recognizes LPS while TLR2 recognizes gram-positive cell components. Additionally, TLR9 recognizes bacterial DNA, TLR5 recognizes bacterial flagellin (47, 48) and TLR3 recognizes double-stranded RNA (49). Recently, it has been shown that LPS from *Porphyromonas gingivalis* is slightly different in structure from *E. coli* LPS and is recognized by TLR2, rather than TLR4 (50). The TLRs also mediate the innate response to fungal infection, specifically; TLR2 and TLR4 have been shown to be involved in the response to *Candida albicans* (51).

Upon TLR activation, the TIR domain mediates interaction of the activated receptor with MyD88, which recruits the IL-1 associated kinase (IRAK) to the receptor complex via death domain interactions. Studies with knockout mice have shown that both MyD88 and IRAK are required for maximal response to LPS stimulation (41). After activation via interaction with the receptor complex, IRAK dissociates from the complex and binds to TRAF6. The IRAK/TRAF6 complex then activates downstream signaling targets, including TAK1, a MAP3K involved in IKK activation after TLR signaling. Activation of TAK1 requires several associated signaling complexes and once activated, TAK1 activates IKKβ and MKK6, resulting in activation of NF-κB, JNK and p38 (52). Thus, TAK1 appears to play a role analogous to that of NIK in TNF-α-induced NF-κB activation, but whether NIK is directly involved in TLR-mediated NF-κB activation is controversial. Activation of NIK by IRAK is known to occur as a result of IL-1β signaling, and there is evidence that TAB1, an adapter protein that interacts with TRAFs and p38, is able to activate NIK via TAK1 (53, 54). However, experiments utilizing adenoviruses expressing dominant-negative IKKβ or NIK proteins showed that NIK isn't necessary for TLR signaling in alveolar macrophages (55). This calls into question whether NIK is critically involved in TLR-mediated NF-κB activation. Although some studies implicate MEKK3 in TLR-mediated signaling, experiments have shown that TAK1 directly phosphorylates IKKβ and is critical for IL-1β signaling via IRAK (52, 56, 57). Thus, the current model for TLR-mediated NF-κB activation involves MyD88-dependent recruitment of IRAK to the activated TLR, association of IRAK with TRAF6, TRAF6 activation, formation of a TRAF/TAK1/TAB1/2 complex and activation of IKKβ. Subsequent phosphorylation of IκB by the activated IKK complex results in NF-κB nuclear translocation (Figure 1).

Hirschfield *et al* demonstrated that TLR2 and TLR4 activation results in activation of different, non-overlapping sets of genes (50). Though the mechanism for this remains unknown, it is possible that different NF-κB dimers are activated, or that additional signaling pathways are activated in parallel to NF-κB, depending upon the upstream signaling molecules activated. In any case, it seems that activation of different TLRs results in different modes of NF-κB signaling that mediate discrete sets of genes required for differential responses to infectious agents. For instance, TLR4 stimulation results in the activation of IL-1β and IFNγ, while TLR2 activates only expression of IL-β (50). In addition to IL-1β and IFNγ, TLR activation results in expression of the TNF-α gene and genes encoding the CXC family chemokines and their receptors (58-60). Since TLR signaling leads to NF-κB-dependent synthesis of TNF-α, IFNα and IL-1β(Figure 1), these cytokines act as feedback inducers of NF-κB, resulting in amplification of the NF-κB-dependent responses to sepsis. Thus, it is likely that TLR-dependent and inflammatory signaling act synergistically to activate a multitude of downstream genes, including NF-κB-dependent genes that affect cell death and organ function.

Despite the importance of the membrane-bound TLRs, NF-κB is also activated in response to molecules from pathogens within the cell. Although, in the case of most intracellular pathogens, the mechanism of this recognition is not well understood, a great deal of work has focused upon the mechanisms by which dsRNA from RNA viruses activates NF-κB. Evidence currently supports two mechanisms for NF-κB activation in response to intracellular dsRNA. The first involves activation of dsRNA-dependent protein kinase (PKR), which activates NF-κB via the IKK signalsome. A second mechanism involves the NOD proteins, which are cytosolic and contain leucine-rich repeats (LRR) similar to the extracellular domains of TLRs. In addition to the LRR domain, which is presumably a PRR, the NOD proteins also have an ATPase domain related to that of APAF-1 and CARD domains enabling NOD proteins to interact with RIP adapter proteins to activate NF-κB (61). NOD1 and NOD2 have also been shown to mediate response to intracellular LPS in the absence of functional TLR4 proteins (62).

Although increased nuclear distribution and DNA binding activity are critical aspects of NF-κB activation, it is clear that modulation of NF-κB's ability to activate gene transcription is independently regulated (63). As is the case for many transcription factors, phosphorylation increases the ability of NF-κB to activate gene expression, either by increasing the activity of the C-terminal transactivation domain of p65, or by modifying interactions between p65 and other transcription factors or co-activators (64). NF-κB phosphorylation occurs in response to several signaling factors, including Akt, PKA, casein kinase II (CKII), Ca^{+2}-calmodulin kinase IV (CaMKIV), TNF-α, IKK and MAPKs (100). For instance, phosphorylation of p65 at position Ser276 is important for interactions between NF-κB and the transcriptional cofactors CBP and p300 (63, 65). Similarly, there is evidence that several of the MAPKs, including p38 and JNK, can phosphorylate nuclear NF-κB and JNK has been found to be physically associated with c-Rel by yeast two-hybrid analysis (66). In neutrophils

subjected to LPS, signaling pathways involving p38 and PI3K are activated, in addition to NF-κB. Blockade of p38α prevented activation of NF-κB and TNF-α synthesis in this model (67). Other studies have shown that p38 blockade results in reduced levels of NF-κB-dependent gene expression, without reduced nuclear levels of NF-κB, in association with reduced phosphorylation of NF-κB coactivator proteins (68, 69). PI3K can potentially activate NF-κB via Akt and IKK-α. IKK-α may work via IκB phosphorylation to increase NF-κB nuclear translocation, or directly to increase NF-κB transactivational activity by phosphorylation of p65 at Ser536 (70-72). To date, results in LPS-stimulated neutrophils support the former mode of regulation (73). Though the relative importance of these differing modes of NF-κB activation and their contributions remain to be determined, it is clear that NF-κB acts as a signaling integrator or "hub" that connects multiple signaling pathways to the regulation of sets of downstream genes that are involved in diverse aspects of the pathophysiology of sepsis.

There is a great deal of evidence supporting multiple feedback regulatory circuits between NF-κB and the signaling pathways that activate NF-κB. For instance, NF-κB is the major regulator of several of the IκB genes, whose products are the direct repressors of NF-κB activation. NF-κB also regulates the expression of TNF-α, the TNF-α receptors (TNFR1 and TNFR2), TRAF1, interleukins, including IL-1α, IL-1β, IL-2, IL-6 and IL-10, and IFNγ. These feedback mechanisms play an important role in potentiation of NF-κB signaling in sepsis, contributing to the production of high levels of inflammatory mediators in multiple cell types, including macrophages and neutrophils. Furthermore, NF-κB is known to activate anti-apoptotic genes in neutrophils, thereby reducing susceptibility to apoptosis and prolonging their acute inflammatory action (74-76). Neutrophils are known to be particularly important in mediating sepsis-induced organ dysfunction. In fact, elimination of neutrophils by immunotherapy or cytotoxic agents prevents development of endotoxemia-induced lung injury in a mouse model (77). Thus, runaway feedback signaling of the innate immune system, involving NF-κB activation and production of further inflammatory molecules could explain both the severity and the NF-κB dependence of many aspects of the pathobiology of sepsis.

5. TOLL-LIKE RECEPTOR SIGNALING, CYTOKINES AND CARDIAC DYSFUNCTION

The effects of sepsis upon the heart have been studied in depth and physiologically include reduced ventricular ejection fraction (both left and right ventricles), increased end-systolic and end-diastolic volumes, elevated heart rate and cardiac output and decreased vascular resistance (78, 79). Although there is general agreement that sepsis leads to systolic dysfunction, the case is less clear for diastolic dysfunction. Recent echocardiographic studies have shown slower LV filling and aberrant LV relaxation, suggesting that compliance is subnormal in septic patients and may contribute to the overall cardiac dysfunction (80-82). There is also evidence of RV systolic and diastolic dysfunction in sepsis, the timing and onset of which correlates with LV dysfunction (83). Patients who do not survive sepsis tend to have lower peripheral vascular resistance, reduced inotropic response to dobutamine and significantly less LV dilatation, relative to survivors (84-86). This has led to the hypothesis that non-survivors, in which compensatory LV dilatation does not occur, succumb to cardiogenic septic shock.

The existence of a myocardial depressant factor in sepsis was first proven by Lefer and later confirmed by Parillo (87, 88). Subsequent studies suggested that the myocardial depressant factor was most likely a protein (89) and recent studies support roles for the cytokines TNF-α and IL-β in myocardial depression associated with sepsis. First, both TNF-α and IL-1β are increased in the serum of septic patients and in animal models of sepsis (90). Second, both cytokines elicit a dose-dependent effect upon myocardial contractility and immunoabsorption of each cytokine partially reverses the depressant effect of human septic serum upon contractility of isolated cardiomyocytes (91-94). However, blockade of either cytokine alone does not completely reverse pathophysiology, and clinical studies using TNFα or IL-1β blocking agents do not show improvement in mortality (95-97). Studies have shown that together, TNF-α and IL-1β act synergistically to depress cardiomyocyte contractility at concentrations 50-100 times lower than either cytokine alone (98, 99, 100) and at levels comparable to those found in sera from septic patients. Experiments performed by Kumar *et al.* demonstrated that immunoprecipitation of both TNF-α and IL-1β from the serum of septic patients eliminates the myocardial depressant activity relative to untreated septic serum (100). These results suggest that TNF-α and IL-1β mediate myocardial depression in sepsis synergistically (83). Interestingly, these same cytokines are known to synergistically activate the downstream transcription factor NF-κB and to be synthesized by an NF-κB-dependent mechanism in response to TLR-activation (101).

Myocardial depression in response to cytokines occurs in two phases, the earliest occurring within minutes of exposure to TNF-α, IL-1β, cytokine combinations or serum from septic animals (100, 102). The second phase occurs within hours and lasts for days (93, 94, 97, 103). Although both phases are thought to involve generation of NO, the early phase appears to involve activation of endothelial NOS (eNOS), while the late phase involves transcriptional activation of the inducible NOS (iNOS) (94, 99, 102, 104-107). Although some studies have shown that pharmacological inhibition of NOS does not affect cardiac depression in endotoxic shock (108, 109), Ullrich *et al.* showed that genetic abrogation of iNOS prevents systolic and diastolic dysfunction after endotoxin challenge (110). Grandel *et al.* showed that the contractile effects of endotoxin on the isolated heart are dependent upon TNF-α synthesis and action, and that the cardio-depressant actions of TNF-α are dependent upon signaling via Cox2 activation and the sphingomyelinase pathway, but are not prevented by NOS inhibition (111). More recently, Ejima *et al.* have shown that $Cox2^{-/-}$ mice are resistant to endotoxin-mediated inflammation and death (11). Thus, it is likely

that multiple end-effectors, including NOS and Cox2, are responsible for the effects of sepsis upon cardiac function.

The myocardium expresses many of the components of the innate immune system. Specifically, the heart is known to express four of the PRRs, CD14, TLR2, TLR4 and TLR6 (112-115). A role for TLR4 in the cardiac response to sepsis has recently been established using mice with a point mutation in *TRL4* (116). Baumgarten *et al.* showed that a *TLR4* mutation, which exists naturally in the C3H/HeJ mice, but not in the related C3HeB/FeJ strain, reduced and delayed the synthesis of myocardial TNF-α and IL-1β after LPS administration. Furthermore, this was associated with reduced activation of NF-κB, reduced synthesis of iNOS and decreased NO production (116). More recently, Nemoto *et al.* showed that the LPS induced cardiac functional depression was largely prevented in C3H/HeJ mice and verified that this was associated with reduced iNOS expression in the heart (117). A related study showed that CD14 is necessary for LPS-induced LV functional depression and for increased TNF-α synthesis (118). Finally, two studies demonstrated that acute activation of NF-κB in the myocardium after LPS administration is blocked in transgenic mice with cardiomyocyte-specific expression of non-degradable IκBα proteins (119, 120). Furthermore, NF-κB blockade in these mice reduces the LPS-induced increase in TNF-α levels and cardiac dysfunction in the isolated heart (121).

Taken together, the studies discussed above demonstrate that a functional innate immune signaling system exists in the heart and TLR4 acts via NF-κB to activate expression of TNF-α, IL-1β, iNOS, and perhaps Cox2. The activity of these inflammatory mediators in the heart explains, to a considerable extent, the functional depression of cardiac function in sepsis. The results of cardiac-specific NF-κB blockade suggest that local blockade of NF-κB operates by 1) reducing production of NF-κB-dependent chemotactic factors that lead to inflammation and infiltration, which would otherwise potentiate the activity of NF-κB and 2) reducing production of NF-κB-dependent gene products which directly affect cardiac function. Thus, although both local and circulating cytokines and chemokines are produced in sepsis, blocking NF-κB in cardiomyocytes disrupts many of the adverse effects of sepsis upon the heart.

6. NF-κB DEPENDENT GENES AS MEDIATORS IN SEPSIS

Activation of NF-κB by TLRs and cytokines plays a role in LPS-mediated lethality and induces infiltration and dysfunction of multiple organs (39, 122-126). Increased levels of NF-κB activation are associated with higher mortality in clinical sepsis (40, 54). Taken together, this suggests that NF-κB plays a central role in sepis in multiple organ systems (39). Although, as discussed above, it is possible that strategies for NF-κB blockade may ameliorate dysfunction of multiple organs in sepsis, it is also true that NF-κB activity is critical for fighting infection, via both the innate immune system and by activation of the adaptive immune system. Systemic NF-κB blockade would be immunosuppressive, a fact borne out by results with p50 knockout mice (128). Thus, therapeutic strategies that block NF-κB or specific sets of NF-κB-dependent genes in a cell-specific manner will likely be required for development of efficacious therapeutic approaches. The central role of NF-κB begs the question of what other NF-κB-dependent genes may be involved in sepsis, in the heart as well as in other organs.

NF-κB is the most thoroughly studied mammalian transcription factor and has been implicated in the regulation of more than 200 genes in a variety of tissue and cell types. The specific subset of genes activated by a particular stimulus depends upon the signaling cascades activated, the number and placement of NF-κB DNA binding sites within specific promoters, the activity of other transcription factors and coactivators and the kinetics of NF-κB activation itself. NF-κB regulates the expression of genes encoding a number of its own subunits, including p65, c-rel, NF-κB-1 and NF-κB-2, as well as the inhibitory IκB proteins, IκBα, IκBβ and IκBγ (129-136, 119, 137-139). Thus, NF-κB signaling can be potentiated by increased levels of NF-κB subunits, or down-regulated by increased synthesis of inhibitors. These feedback mechanisms are likely to be extremely important in the amplification of the response to sepsis. Peripheral blood mononuclear cells (PBMCs) from patients that die as a result of sepsis have increased levels of NF-κB activation relative to survivors (39). An extensive list of NF-κB regulated genes can be found at http://people.bu.edu/gilmore/nf-kb/target/index.html, maintained courtesy of Dr. T. Gilmore. From this list it is apparent that NF-κB can potentially affect a wide variety of biological systems and processes.

NF-κB-dependent genes that have been implicated in the pathogenesis of sepsis include the cytokines TNFα and IL-1β, which, as discussed previously, are pro-inflammatory and involved in regulating the activation of NF-κB both systemically and in the heart. In addition to cytokines, there is also support for NF-κB-dependent expression of factors, such as P-selectin, E-selectin, iNOS, heme-oxygenase-1 (HO-1), C-reactive protein, tissue factor and Cox2, in sepsis. In addition to it cardiac-specific effects (above), there is evidence that iNOS plays opposing roles in sepsis. Specifically, NO and iNOS have been implicated in inhibition of endothelial adhesion and thus neutrophil migration in sepsis, however, NO is also implicated in antimicrobial activity of neutrophils. NO and iNOS are also implicated in endothelial dysfunction and in hypotension that results from sepsis (140, 141). Benjamin *et al.* observed decreased mortality and restoration of neutrophil migration in septic mice (cecal ligation/puncture model) treated with aminoguanidine (AG), a relatively specific iNOS inhibitor (142). In the same study iNOS knockout mice showed normal neutrophil migration, but 100% mortality. Similar results were obtained by the same group after sepsis induced by i.p. injection of *S. aureus* (143). These investigators concluded that NO was responsible for defective neutrophil migration, thus inhibition by AG

resulted in restoration of neutrophil migration. In iNOS knockout mice however, the lack of antimicrobial NO production by neutrophils likely contributes to mortality, despite normal neutrophil migration. A study by Hollenburg *et al*. demonstrated decreased mortality in iNOS knockout mice subjected to cecal ligation/puncture (141). This study showed that iNOS abrogation in a sepsis model improved microvascular catecholamine responsiveness, suggesting that iNOS plays a role in the pathophysiology of hypotension and decreased vasopressor response. Similarly, Kristof *et al*. showed that iNOS knockout mice are more resistant to LPS-induced pulmonary injury relative to wild type mice (144). On the other hand, studies by Benjamin *et al*. and by Cobb *et al*. demonstrated increased mortality in iNOS knockout mice using a cecal ligation/punture model of sepsis (142, 145), while several other studies found no significant difference in mortality between iNOS knockout and wild type mice (146, 147) after LPS-induced sepsis. The different outcomes of these studies may be somewhat dependent upon the details of the models employed, and may involve multiple antithetical effects of NO during sepsis. Thus, the overall role of iNOS in sepsis remains somewhat controversial.

Enhanced formation of NO is implicated in myocardial depression associated with sepsis (148). There is evidence that NO uncouples the β-adrenergic (βAR) system by phosphorylation of the voltage-dependent calcium channel (VDCC). This has direct effects upon calcium handling via the inhibition of excitation-contraction coupling, decreasing the phosphorylation of phospholamban (PLN) and decreasing the expression of both PLN and the sarcoplasmic reticulum calcium channel (SERCA2a), thereby modulating calcium uptake (149-151). Decreased calcium uptake reduces the calcium transient and thus cardiomyocyte contractility. NO also decreases the phosphorylation of TroponinI with concomitant reduction of contractile force production (152, 153). Baumgarten *et al*. determined that myocardial nitrate levels and iNOS protein levels increase 8 and 12 hours after LPS injection, that this is associated with NF-κB activation 30-60 minutes after injection and that both NF-κB activation and the increase in iNOS are TLR4-dependent (116). A role for NO in myocardial dysfunction associated with sepsis was also shown by Ullrich *et al*. who demonstrated that iNOS knockout mice have normal ventricular function after LPS administration (110). Thus, there is a direct effect of NO generated consequent to iNOS gene expression in myocardial dysfunction associated with sepsis.

A role for Cox activity in sepsis was suggested by an early study in which ibuprofen, a relatively non-specific, non-steroidal anti-inflammatory drug (NSAID) was found to reduce mortality associated with endotoxemia (154). Subsequent studies showed that ibuprofen was protective against hypotension, acidosis and depression of cardiac function associated with sepsis (155, 156). Liu *et al*. showed that Cox2 RNA levels were significantly increased in the heart and lungs from rats treated with LPS (157). A seminal study by Reddy *et al*. demonstrated that a relatively specific inhibitor of Cox2, NS398, reduces the increase in PGE(2), a major product of Cox2 activity, and decreases short-term mortality in mice treated with LPS (158). A more recent study supports this result (159). The pro-inflammatory and morbid activity of Cox2 is supported by a study subjecting Cox2 knockout mice to an endotoxin model of sepsis (11). These investigators showed that Cox2 deficient mice displayed improved survival, reduced/delayed expression of iNOS and HO-1 and reduced/delayed activation of the transcription factors NF-κB and AP-1, implicated in transcriptional regulation of these genes. Interestingly, IL-10, an anti-inflammatory cytokine, was increased in Cox2 knockout mice, and these authors hypothesize that IL-10 is reduced by prostaglandins (Figure 2) and is responsible for the reduction of iNOS and HO-1 levels via reduced activation of NF-κB and AP-1. Thus, Cox2 has overall injurious effects in sepsis and may be involved in mediating a balance between pro- and anti-inflammatory mediators that critically regulate the overall outcome.

HO-1 is an inducible cytoprotective enzyme that is upregulated subsequent to oxidative stress (160). The activity of HO-1 is responsible for degradation of heme, resulting in the generation of biliverdin, iron and carbon monoxide (CO). Although primarily implicated as an adaptive stress-response protein, recent studies have shown that HO-1 and its product CO have anti-inflammatory properties. HO-1 has been shown to be beneficial and its inhibition deleterious to rats submitted to hemorrhagic shock (161). Humans deficient in HO-1 are sensitive to oxidative injury (162) and studies with embryonic fibroblasts from HO-1 knockout mice demonstrate that HO-1 activity is protective against oxidative injury (163). One study found that HO-1 activity contributes to liver dysfunction in sepsis by up-regulation of cGMP (164) and is also involved in the endotoxin-induced hypotension that accompanies sepsis (165). However, multiple studies support that HO-1 is protective in LPS and cecal ligation/puncture models of sepsis (161, 166, 167). Tamion *et al*. found that pre-treatment with heme to induce HO-1 expression reduced TNF-α levels after induction of hemorrhagic shock (161). The hypothesis that CO is responsible for the anti-inflammatory effects of HO-1 activity is supported by a study presented by Otterbein *et al*., who demonstrated that administration of exogenous CO, at biologically relevant levels, inhibits production of TNF-α and enhances synthesis of IL-10 in macrophages. Furthermore, inhaled CO reduced serum IL-1β and IL-6 levels and enhanced survival of mice after administration of a lethal dose of LPS (168). Studies using JNK1 and JNK2 knockout mice demonstrate that HO-1 induction by LPS occurs via a MAPK-dependent mechanism that involves, at least in part, activation of AP-1 (169, 170). Although there is no direct proof that NF-κB directly modulates HO-1 gene expression during sepsis, the involvement of JNK and AP-1, both of which interact with NF-κB, and previous evidence that HO-1 activation subsequent to TNF-α signaling involves NF-κB (171), is suggestive. Yet, much as in the case of iNOS, HO-1 appears to mediate both beneficial and injurious effects in sepsis. In fact, CO and NO interact in many ways. NO is itself a stimulus for HO-1 gene expression and there are several ways in which HO-1 or its product, CO, reduces NO production (172).

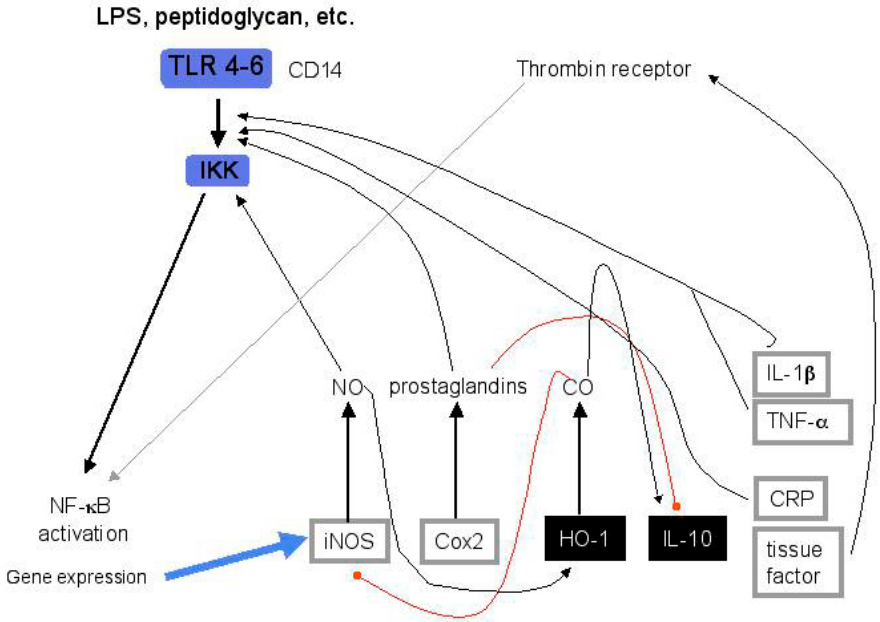

Figure 3. NF-κB-dependent gene products mediate positive and negative feedback that influences septic signaling in the heart and other organs. Activation of NF-κB results in NF-κB-dependent gene expression. The genes activated are generally pro-inflammatory or pro-coagulation (grey open boxes; iNOS, Cox2, TNF-α, IL-1β, C-reactive protein (CRP) and tissue factor), or are anti-inflammatory (black boxes; IL10 and heme-oxygenase-1 [HO-1]. Several of the pro-inflammatory gene products mediate positive feedback, amplifying the TLR- and cytokine-mediated signaling (black arrows). The anti-inflammatory gene products act in opposition to the pro-inflammatory gene products and prostaglandins repress IL-10 (red lines).

Endotoxins and inflammatory responses produce pro-coagulation effects that contribute to the pathophysiology of sepsis by stimulating intravascular coagulation producing thrombosis and hemorrhage. NF-κB-dependent genes associated with this aspect of sepsis include tissue factor and Protein C. Tissue factor activation during sepsis can result in production of thrombin and fibrin from prothrombin and fibrinogen, respectively. A study by Bohrer *et al*. demonstrated that inhibition of NF-κB activation in a murine endotoxemia model, using an IκB-overexpressing plasmid, reduces induction of tissue factor in association with reduced mortality after LPS administration (39). Increased tissue factor synthesis is associated with mobilization of cryptic tissue factor, increased fibrin deposition and increased formation of thrombin-antithrombin III complexes. Thus, NF-κB-mediated expression of the tissue factor gene is critical to the development of sepsis-related coagulation. In addition, binding of thrombin to its receptor activates NF-κB and iNOS, which can potentiate signaling in sepsis (Figure 3). Sepsis and inflammatory signaling also inactivates fibrinolysis in concert with activation of tissue factor, which serves to further increase coagulation. Recombinant Protein C, an activated version of the endogenous inhibitor of thrombin formation, has been shown to modulate NF-κB activity and attenuates the inflammatory response and apoptosis in endothelial cells and monocytes (173).

C-reactive protein (CRP) is a nonspecific acute-phase serum protein that has been shown to be a sensitive marker of tissue inflammation, infection and damage. CRP is a member of the pentraxin family of calcium-dependent ligand-binding plasma proteins and binds to a variety of ligands, resulting in the aggregation or precipitation of the ligands, which include phosphocholine, modified plasma lipoproteins, damaged cell components, as well as glycans, phospholipids and proteins from pathogenic organisms. It is unclear whether CRP is a marker or plays a functional role in inflammation. When bound into aggregates, CRP may activate complement and it has been suggested that CRP may act as a proinflammatory mediator in specific situations (174). High serum levels of CRP are associated with a poor outcome after MI and acute coronary syndromes. CRP is an NF-κB-dependent gene that is activated by cooperation between NF-κB and C/EBPβ in response to IL-6 and IL-1β (175, 176).

Heat shock proteins are, in general, cardioprotective (177, 178). The heat shock response is known to mitigate sepsis-induced mortality, cardiovascular dysfunction and apoptosis (179, 180). de Vera *et al*. showed that induction of the heat shock proteins (HSPs) blocks the cytokine-induced expression of iNOS and production of NO in rat hepatocytes (181). More recently, Yang *et al*. showed that NF-κB activation in a cecum ligation/puncture model is modified by heat shock response (180). This study showed that NF-κB was activated early in lymphocytes after intiation of sepsis, but suppressed during late stages. Prior heat shock prevented the late suppression of NF-κB activity in association with increased HSP72 expression. A connection between HSPs and cytokine signaling has been made in infectious disease,

inflammation, lymphoma and in the heart (182-185). The possibility that preservation of late NF-κB activation shifts the equilibrium between pro- and anti-inflammatory aspects of signaling in sepsis is supported by studies demonstrating that heat shock inhibits levels of the cytokines TNF-α and IL-1β as well as early NF-κB activation by sepsis (179). This phenomenon seems to involve stabilization and increased levels of the NF-κB inhibitor IκBα, which may be a novel heat shock protein in its own right, possessing a potential heat shock responsive element it its promoter (186, 187). HSP70-family heat shock proteins are known to block NF-κB activation via two mechanisms, inhibition of IKK activity (188) and direct retention of NF-κB in the cytoplasm by physical interaction with HSP 70 (189). Thus, heat shock may act directly upon NF-κB activation to modulate the balance between pro- and anti-inflammatory as well as pro- and anti-apoptotic signaling during sepsis. Interestingly, activation of the heat shock response after initiation of sepsis leads to the paradoxical result that injury, including apoptosis, is increased (190).

No discussion regarding heart disease is complete without discussion of programmed cell death, or apoptosis. In fact, apoptosis has been shown to play a role in sepsis and specifically in the cardiac dysfunction that accompanies sepsis. Several studies have shown that caspase inhibition reduces the myocardial dysfunction that occurs subsequent to endotoxin administration (191, 192). It has been shown that LPS induces both pro- and anti-apoptotic pathways, in a CD14 and NF-κB-dependent fashion, in endothelial cells (101) Comstock *et al*. showed that LPS induced TNF-α release is involved in apoptosis of cardiomyocytes that may contribute to cardiac dysfunction (112). Endotoxin infusion in rats induces both pro- and anti-apoptotic pathways. McDonald *et al*. showed endotoxin induces early pro-apoptotic changes in Bax and Bcl-2 protein levels, but modulates gene expression such that this is reversed by 24 h post-infusion (193). These changes correspond to increased caspase activation and TUNEL positive cells in the myocardium 24 h after endotoxin exposure. Since Bcl2 activates NF-κB via the phosphorylation and degradation of IκB (194) cross-talk regulation must exist between NF-κB, pro- and anti-inflammatory signaling pathways. It is evident that the effects that NF-κB has upon apoptosis are tissue-specific. A study by Joshi *et al*., using a cecal ligation/puncture model of sepsis, showed that pharmacological inhibition of NF-κB activation had no effect upon apoptosis in thymocytes or phagocytes, but caused a significant reduction in apoptosis in Peyer's patch B cells. Thus, the effect of cell death in sepsis is likely tissue- and organ-specific (195), a fact that will likely complicate development and usage of anti-apoptotic therapies.

It has become obvious that genes involved in opposing responses, such as pro- and anti-apoptotic, pro- and anti-inflammatory responses, and cell growth and division, are activated by NF-κB in sepsis. It is possible that co-activation of cell growth/survival pathways and pathways activating cidal genes may have evolved to mitigate the effects of the latter upon host cells, while allowing them to kill invading microorganisms. Regardless, it has critical implications for understanding the role of NF-κB in pathophysiology and for developing therapies based upon inhibition of NF-κB and NF-κB-dependent gene expression.

7. PROSPECTUS AND CLINICAL RELEVANCE: AN EVER-EXPANDING PARADIGM?

Sepsis has been described as a disequilibrium syndrome, involving imbalance between pro- and anti-inflammatory responses (196). Specifically, sepsis induces the pro-inflammatory cytokines TNF-α and IL-1β, as well as the anti-inflammatory cytokines IL-10 and TGF-β. Both pro- and anti-inflammatory pathways have destructive aspects and are networked such that it is difficult to affect one without the other. Furthermore, the signal transduction pathways involved in sepsis cross-talk at several levels and, as discussed, both positive and negative feedback interactions between end-effectors and upstream components come into play. In fact, several of the mediators of inflammation and cell death that play a role in sepsis, including TNF-α and NF-κB, are known to be involved in both beneficial and injurious aspects of pathophysiology. Finally, the effect of a particular signaling component depends greatly upon the cellular environment and activation state of networked pathways. This leads to the possibility that the same factor may play a very different role in different cell types, or even in the same cell type depending upon the stage of disease or the order in which insults occur. For instance, heat-shock administered prior to endotoxin challenge is protective against apoptotic cell death, while reversing the sequence of insults results in enhanced apoptosis (148, 190, 197). There is evidence that the effect of the sequence of stimuli may involve heat shock modulation of NF-κB activation (190). The overall action of NF-κB may thus depend upon the combinations of upstream stimuli that are active and the sequencing of stimuli. How NF-κB activation results in transactivation of discrete sets of genes that underlie specific pathophysiological phenomena remains incompletely understood. It is likely that this involves the action of multiple transcription factors, activated in parallel and interacting with NF-κB, as has been shown in several other situations (198, 199). Thus, understanding the detailed mechanism of inflammatory and cell death/survival signaling in sepsis is necessary for the development of successful therapies. This new paradigm for understanding the action of NF-κB and related effectors in sepsis is in agreement with the results of clinical trials for sepsis.

Clinical studies to date have focused upon neutralization of endotoxin, inhibition of recognized effector molecules, most of which we have discussed above, or administration of anti-inflammatory molecules (6). Nine trials have been conducted to test the effects of neutralizing endotoxin using antibodies; only three of these studies showed significant benefit and the pooled results show no significant benefit. Ten trials of TNF-α blocking therapies show a small but significant reduction in mortality (6) although trials of a recombinant p75 fusion protein resulted in increased mortality

(129, 200). Three trials have investigated the effect of blocking IL-1β using a recombinant soluble IL-1R protein. One of these studies showed a significant reduction in mortality, while two showed no such effect (201-203). Several small studies using inhibitors of iNOS showed reduction of hypotension. However, inhibition of iNOS was found to be associated with reduced cardiac output and increased pulmonary artery pressure, and a phase III clinical trial of N(G)-monomethyl-L-arginine (L-NMMA), a NOS inhibitor, was discontinued due to increased mortality (unpublished, reviewed in reference 6). Two studies employed strategies to block coagulation using activated Protein C and antithrombin, both of which block thrombin formation. In both cases, a significant reduction in mortality was observed. Although a phase II study of tissue factor pathway inhibitor (TFPI) showed a trend towards reduced mortality, this result could not be repeated in a larger phase III trial (6). Although trials using corticosteroids prior to 1990 showed little effect, more recent trials using a combined corticosteroid treatment, hydrocortisone and fludrocortisone, showed a significant 10% reduction in mortality (204). The most efficacious therapy thus far appears to be fluid resuscitation. A recent study showed that aggressive fluid resuscitation resulted in a significant 16% reduction in mortality (6, 205). Thus, targeting the proposed mediators in sepsis has not proven terribly effective to date. This could be due to a number of reasons, some of which derive from the considerations discussed above. First of all, therapy could be provided at inappropriate timepoints during development of sepsis. Most studies using animal models have been performed by application of blockade prior to initiation of sepsis, whereas treatment of clinical sepsis is after the occurrence of symptoms. Considering the previously discussed effect of sequence stimuli regarding heat shock and sepsis, perhaps it should come as no surprise that the results are contradictory. Second, patient populations are heterogeneous, and, as discussed, secondary insults and disease can have effects upon pro- and anti-inflammatory or other signaling pathways that may affect those active in sepsis. Third, animals are imperfect models for human sepsis and there is a great deal of variability in the models used for animal experimentation. Fourth, therapeutic modulation of single mediators is problematic in sepsis since a large number of mediators have been identified. Combination therapy may be required to achieve efficacious treatment. Though evidence from animal experiments supports that proposition that co-inhibition of the cytokines TNF-α and IL-1β is more beneficial than either treatment alone (91, 206), such therapies have not yet been subjected to clinical trials. Fifth, the current inability to target specific cell types with therapies is detrimental, since studies have shown that the same signaling pathways may have opposite effects upon the same endpoint in different cell types; for instance, the effect of NF-κB blockade upon apoptosis in thymocytes vs. Peyer's patch B cells (195). NF-κB activation occurs in multiple organs and cell types, and may be primarily protective in one tissue but injurious in another. Thus, a detailed understanding of the molecular basis of the pathophysiology of sepsis is needed, as is the technology to deliver or restrict the action of therapeutic molecules to specific cell types and/or organs. The hope is that, with the development of new knowledge and technology, we will be able to specifically block pro-inflammatory and pro-apoptotic signaling in the heart for instance, while avoiding adverse effects in other organs and drastically improve the outcome of sepsis in a clinical setting.

8. CONCLUSIONS

NF-κB is involved in regulating opposing responses, such as pro- and anti-apoptotic, pro- and anti-inflammatory responses, and cell growth and division in sepsis. This situation has profound implications for understanding the role of NF-κB in disease states and for developing therapeutic interventions that ameliorate the injurious aspects while retaining the protective aspects of NF-κB signaling. This is further complicated by the fact that NF-κB activation in sepsis occurs in multiple organs and cell types, and may be primarily protective in one tissue but injurious in another. How do we hope to make sense of the morass of biological effects that NF-κB can potentially mediate? It seems likely that specific stimuli result in specific modes of NF-κB activation that are characterized by modifications of NF-κB (dimer composition, phosphorylation, nitrosylation, acetylation) that, with or without cooperative regulation of other transcription factors, result in regulation of discrete sets of NF-κB dependent genes. Methodical assessment of NF-κB activation kinetics and NF-κB-dependent gene expression and the phenotypes associated with blockade or ablation of these gene products is needed to define the specific actions of NF-κB and the role of NF-κB dependent gene expression in specific physiological and pathophysiological states.

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