A novel adenovirus mediated gene therapy approach improves survival in a murine model of endotoxic shock

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Objectives and Background: During the course of sepsis, pro-inflammatory cytokines such as TNFα are produced in large amounts leading to multiple organ failure and ultimately death. Excessive TNFα production during sepsis is largely dependent on the activation of NFκB pathways by lipopolysaccharide (LPS), a major sepsis-inducing agent of gram negative infections. In the liver, activation of NFκB has been associated with protective anti-apoptotic pathways under conditions of hepatocellular growth and regeneration. Under pro-inflammatory conditions, however, it remains controversial whether NFκB predominantly plays a protective or deleterious role in liver injury.

Methods: Using recombinant adenoviruses expressing dominant negative mutants to either IKKα (Ad.IKKαKM) or IKKβ (Ad.IKKβKA) we have investigated the mechanisms of NFκB activation and its influences in determining hepatocellular fate in the liver following a lethal dose of endotoxin in a murine model.

Results: Ectopic IKKβKA expression in the liver significantly improved the survival of mice (p<0.0001, 25% survival at 12 days) from endotoxic shock (which normally kills 50% of mice by 1 day and all mice by 3 days post-LPS injection). No significant difference in survival was noted between uninfected and Ad.EGFP or Ad.IKKαKM infected animals. This improvement in the survival rate was correlated with a reduction in NFκB activation, serum TNFα and ALT levels, and hepatocellular apoptosis following LPS exposure.

Conclusion: These results demonstrate that NFκB activation predominantly plays a pro-apoptotic role in the liver under conditions of endotoxic shock and suggests new therapeutic strategies for intervention.

Key words: LPS, TNF, NFκB and endotoxic shock


Introduction

The sepsis syndrome is associated with a systemic inflammatory response to an infection with a mortality rate of more than 100,000 people each year in the United States alone.1-3 Gram-negative bacteria are the main cause of sepsis and lipopolysaccharide (LPS), a component of the gram-negative bacterial cell wall, plays a major role in the pathogenesis of sepsis.4,6 Consequently, LPS and gram-negative infection induce similar pathophysiologic responses in animals.1 During the course of sepsis, the overwhelming production of proinflammatory cytokines such as tumor necrosis factor alpha (TNFα) leads to the systemic inflammatory response syndrome (SIRS).8-10 Moreover, TNFα has been shown to be the major player in endotoxin-
induced lethality during the course of sepsis.\(^{11,12}\) Hence, organ-derived TNFα plays a crucial role in the development of multiple organ dysfunction syndrome (MODS).\(^ {13}\) Together, these results emphasize the importance of TNFα in the development of the sepsis syndrome. Numerous animal studies have been conducted to understand the pathophysiology of sepsis. For example, mice lacking tumor necrosis factor receptor 1 (TNFR1) were found to be resistant to endotoxin.\(^ {14,15}\) In addition, a passive immunization with anti-TNF antiserum or soluble recombinant TNF receptor was useful in providing protection against LPS.\(^ {16,17}\) More recently, replication defective adenoviruses carrying potentially therapeutic genes have been evaluated as an approach to treat endotoxic shock. One such recombinant adenovirus vector expressing the human TNF receptor extracellular domain\(^ {18}\) effectively neutralized LPS induced TNF production.\(^ {19}\) Additionally, adenoviral mediated gene transfer of the LpsR/Ran gene into primary macrophages obtained from endotoxin sensitive mice reduced LPS induced TNF production in vitro by an unknown mechanism.\(^ {20}\) These studies suggested that down regulation of TNFα production or its neutralization might prove useful in the treatment of sepsis.

LPS induced TNFα production is largely dependent on NFκB activation.\(^ {21-25}\) NFκB nuclear translocation, an early step in activation, is regulated by two IKK Kinases, IKKα (IKK-1) and IKKβ (IKK-2).\(^ {26-28}\) A direct link between TNFα induction and endotoxin-induced death has been clearly established. However, the in vivo role of NFκB in producing a pro-inflammatory state during endotoxic shock while at the same time modulating cell survival has remained unclear. For example, although NFκB is known to induce TNFα gene expression following LPS exposure,\(^ {21-25}\) NFκB activity has also been shown to be an important anti-apoptotic factor during liver regeneration.\(^ {29-31}\) The manner by which NFκB balances the induction of pro-inflammatory and anti-apoptotic effects remains unclear. To this end, we have evaluated the potential of two recombinant adenoviruses expressing dominant negative mutants to either IKKα or IKKβ\(^ {32}\) to selectively block the deleterious effects of TNFα during the course of experimentally induced lethal endotoxic shock. Results from these studies suggest that under pro-inflammatory conditions such as endotoxicosis, NFκB activation predominantly plays a pro-apoptotic role in the liver through the induction of TNFα.

**Materials and Methods**

**Production of recombinant adenovirus constructs**

Four recombinant adenoviral vectors expressing Enhanced Green Fluorescent Protein (Ad.EGFP), a dominant negative mutant form (K44M) of IKKα (Ad.IKKαKM), a dominant negative mutant form (K44A) of IKKβ (Ad.IKKbKA), and a luciferase reporter gene driven by NFκB transcriptional activation (Ad.NFκBLuc), were used for functional studies. Generation of Ad.IKKαKM, Ad.IKKβKA, and Ad.NFκBLuc constructs were described elsewhere.\(^ {25}\) Recombinant adenoviral stocks were generated as previously described\(^ {33}\) and were stored in 10 mM Tris with 20% glycerol at –80°C. The particle titers of adenoviral stocks were typically 10^13 DNA particles/ml. The functional titers of adenoviral stocks were determined by plaque titering on 293 cells and expression assays for encoded proteins. Typically the particle/pfu ratio was equal to 50. Male (30 g) athymic nu/nu mice (Harlan Sprague-Dawley) were used for the assays. Viral infections were performed by tail vein injection with 10^11 particles of purified virus in 100 µl of saline.

**LPS model of murine sepsis**

Lipopolysaccharide (LPS) isolated from E. coli serotype 055:B5 (L-2880) was purchased from Sigma (St. Louis, Missouri) and used as an endotoxin to induce lethal endotoxic shock in nu/nu mice. LPS was dissolved in PBS and injected IP into mice (40 µg/gbw). Mice exhibited classic symptomatic signs of sepsis (weak, febrile, lethargic and anorexic) within a few hours following LPS injections. The criteria for the hypodynamic phase of sepsis using the rat endotoxic shock model were established by monitoring mean arterial pressure (MAP) following LPS administration.\(^ {34}\) Shock was inferred from a decrease in blood pressure over the course of a 24 hr period. Carotid arterial catheters were placed 24 hrs before the measurements in order to avoid surgical effects such as anesthesia on the data. MAP was continuously recorded 30 minutes prior to and up to 24 hrs following LPS treatment as described previously (n=3).\(^ {37}\) MAP of nude mice decreased from 104 ± 5.4 mmHg to 65 ± 3 in 24 hrs following LPS. However, the reduction in MAP was significant as early as 3 hours following LPS administration. This level of reduction (≥ 40 mm Hg) is very similar to what is described as sepsis-induced hypotension.
Liver apoptosis assay

Animals were perfused with 2% paraformaldehyde and livers were infiltrated with 30% sucrose prior to embedding in O.C.T. Compound by quick freezing (Tissue-Tek, Elkhart, Indiana) and sectioning. Sections were treated with 2% paraformaldehyde for 30 minutes followed by a 10 min post-fixation in ice-cold methanol. Sections were then permeabilized in 0.01% Triton-X-100. The in situ cell death detection kit, TMR red (Boehringer-Mannheim), was used to detect apoptotic cells in liver sections and results were analyzed by fluorescent microscopy. Image quantification was performed using Image-Pro Plus 4.1 program from Media Cybernetics (Silver Spring, Maryland) to determine the percent apoptotic area. All other statistical analyses (survival etc.) were performed using the Prism program from GraphPad Software Inc. (San Diego, California) and are specifically stated in the figure legends.

Results

LPS induced NFκB activity is down regulated by ectopic expression of a dominant negative mutant to IKKβ. With an overall goal to evaluate whether NFκB induction during the course of endotoxic shock plays a deleterious or protective role in survival, we first sought to establish the effectiveness of inhibiting NFκB activation through the IKK complex in vivo. Two recombinant adenoviral vectors expressing dominant negative forms to IKKα (Ad.IKKαKM) and IKKβ (Ad.IKKβKA) were used for these studies. To avoid complications induced by cellular immune responses to E1-deleted recombinant adenovirus,59 nu/nu athymic mice were used. These mice demonstrated stable high-level transgene expression in the majority of hepatocytes two weeks after tail vein infection (Figure 1A). Additionally, the two-week lag time after infection also allowed recovery from any acute liver injury that might have resulted from adenoviral infection. Western Blot analysis of liver lysates obtained from Ad.IKKαKM and Ad.IKKβKA infected animals indicated that both transgenes were expressed at equal levels in the liver (Figure 1B). In order to assess the functional ability of the Ad.IKKαKM and Ad.IKKβKA constructs to inhibit LPS induced NFκB activation, mice were co-infected with an adenovirus vector carrying a luciferase reporter gene under the control of NFκB regulatory sites (Ad.NFκBLuc). Only Ad.IKKβKA infection significantly reduced LPS induced NFκB activity following LPS exposure (Figure 1C). No significant reduction was obtained when animals were infected with Ad.EGFP or Ad.IKKαKM. Because TNFα production is largely dependent on NFκB activity,23-25 these results suggested that the Ad.IKKβKA construct is best suited to attenuate LPS induced TNFα production in vivo.

Ectopic expression of IKKβKA, but not IKKαKM, improves survival from endotoxic shock. After establishing the parameters for efficient gene delivery of IKK mutants to the liver, the effects of IKKαKM or IKKβKA expression on the survival of nude mice were evaluated following a lethal dose of LPS (40 µg/gbw). As seen

in Figure 2, Ad.IKKαKM or Ad.EGFP infection did not alter the survival curve following LPS challenge over the course of two weeks (50% death occurred by 36 hrs and all mice were dead by 96-120 hrs). This was not significantly different from survival in the uninfected LPS treated group (50% death by 24 hrs and all mice were dead by 72 hrs). In contrast, Ad.IKKβKA administration significantly improved the survival rate of these animals at a level of 25% by 12 days (at which time the experiment was terminated). These data demonstrate
that IKKβKA expression in the liver decreases LPS-induced lethality in nude mice.

Ad.IKKβKA infection in the liver selectively attenuates LPS induced serum TNFα and alanine aminotransferase (ALT) levels. Previous studies have shown that serum TNFα levels correlate with the severity of sepsis in humans.9,10 In order to account for the therapeutic effect of IKKβKA expression, serum TNFα levels were analyzed by ELISA following LPS administration. Maximum elevation in TNFα levels was detected three hours following LPS challenge in mice (Figure 3A). At the same time, a significant reduction in the mean arterial pressure (a drop from 104±5 mmHg to 76±6 mmHg) was observed as early as three hours following LPS administration (data not shown). The expression of IKKβKA, but not IKKαKM, dramatically reduced LPS induced TNFα levels in these mice (Figure 3A). This reduction was correlated with a decrease in LPS induced NFκB activation (Figure 1C). In order to assess the extent of liver damage caused by endotoxin, serum ALT levels were analyzed (Figure 3B). Maximum elevation in serum ALT levels was detected three hours following LPS exposure and persisted for up to twelve hours. No reduction in serum ALT levels was observed in Ad.EGFP or Ad.IKKαKM infected animals. In contrast, Ad.IKKβKA infected animals exhibited a significant reduction in serum ALT levels at all time points following LPS exposure (Figure 3B). These results suggest that IKKβKA expression not only reduces LPS induced TNFα production but also decreases liver damage caused by endotoxin.

Hepatocellular apoptosis induced by endotoxin is ameliorated by ectopic expression of the dominant mutant IKKβKA. Given the previously reported anti-apoptotic effects of IKKβ and NFκB under conditions of liver regeneration,29-32 we sought to better understand how inhibition of IKKβ and NFκB activation could so dramatically improve survival following a lethal dose of endotoxin. Based on our findings, we reasoned that inhibition of IKKβ and hence NFκB, might improve sur-

Figure 2
IKKβKA expression in the liver increases survival following experimentally induced lethal endotoxic shock. Nude mice were infected with either Ad.EGFP, Ad.IKKαKM or Ad.IKKβKA constructs, two weeks prior to IP LPS challenge at 40 µg/gbw. The survival was monitored every 12 hrs following LPS administration for a total of 12 days. Each curve represents the data obtained from 16 animals (n=16) with the exception of the PBS controls (n=8). The X-axis represents hours after LPS administration. The survival rate is expressed as the percent survival on the Y-axis. Statistical analysis of survival curves using the Logrank test indicates that there is a significant difference in the survival rate of Ad.IKKβKA infected animals (P<0.0001).
Hepatic expression of the dominant negative mutant to IKKβ (IKKβKA) decreases LPS induced serum TNFα levels and serum ALT levels following lethal endotoxin. Mice were infected with adenoviral constructs as indicated to the right of panel A, two weeks prior to LPS administration. Sera were collected at different time points following LPS injections as indicated on the X-axis. TNFα concentrations shown in panel A were detected by an Elisa assay. Each bar represents a Mean (+/- SEM) of n=6 independent data points. The effect of IKKβKA expression on LPS induced serum ALT levels is shown in panel B. Mice were injected with various recombinant adenoviral constructs as indicated in panel A. Serum ALT levels were determined as described in Materials and Methods and values represent the mean (+/- SEM) of n=6 independent animals (same animals shown in panel A). Assays in panels A and B were repeated twice to confirm the observations.

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**Figure 3**

Hepatic expression of the dominant negative mutant to IKKβ (IKKβKA) decreases LPS induced serum TNFα levels and serum ALT levels following lethal endotoxin. To directly evaluate the contribution of inhibiting IKKβKA on liver apoptosis following endotoxin exposure, TUNEL assays were performed. In the absence of LPS administration, no significant degree of hepatocellular apoptosis was detected in livers infected with any of the adenoviral constructs (Figure 4). IKKβKA expression reduced liver apoptosis (3-fold) in endotoxin-exposed animals from that seen in uninfected LPS-treated controls (4.3%). Not surprisingly, IKKαKM or EGFP expression did not appear to affect LPS induced liver apoptosis. In summary, these results suggest that IKKβKA expression can attenuate LPS induced NFκB activation, serum TNFα and ALT levels, and protect mice from LPS induced liver damage and apoptosis leading to improved survival.
Discussion

Sepsis remains a very serious medical problem with a high mortality.2,3 This is partially due to our incomplete understanding of the pathophysiologic mechanism(s) of sepsis, which involves a very delicate regulation of both inflammatory and anti-inflammatory responses.8,40 Various laboratory models of sepsis have been described.30 Among these, the endotoxin model involving LPS injection into mice has been one of the most commonly used methods of studying both the hypodynamic and the hyperdynamic phases of sepsis.8,41 Despite concerns raised regarding the relevance of this

Figure 4

Ad.IKKβKA infection of the liver reduced LPS induced liver apoptosis. Animals were injected with adenoviral constructs expressing EGFP (panel B), IKKαKM (panel C) or IKKβKA (panel D) or PBS alone (panel A) two weeks prior to LPS injections. Liver tissue was harvested 24 hrs following LPS injection and later embedded in O.C.T. Compound. Apoptosis was detected on liver sections using the in situ TUNEL assay system as described in Methods. Right panels indicate liver sections of LPS injected animals. Left panels are PBS controls. Livers of four different animals analyzed for each condition are shown. A quantification of the apoptosis data is presented in panel E. Values represent the Mean ±SEM percent apoptotic area for n=4 animals. ANOVA followed by Bonferroni’s Multiple Comparison Test was used for statistical analysis with p<0.05 as significant. † marks statistically significant differences compared to uninfected LPS treated animals. Ad.EGFP and Ad.IKKαKM infected groups had p>0.05 when compared to uninfected LPS treated animals.
model of experimental sepsis to the clinical form seen in humans, this model has been widely accepted as a valid laboratory tool for studying mechanisms of sepsis.

During the course of endotoxic shock, proinflammatory cytokines are produced in excessive amounts leading to endotoxic shock and death. The most important of these cytokines appears to be TNF α, and elevated levels of serum TNF α have been directly correlated with the severity of sepsis in humans. In animal models, directly targeting this cytokine with TNF neutralizing antibodies or a soluble TNF receptor have been demonstrated to be beneficial in reducing the severity of endotoxic shock. Furthermore, mice deficient in TNF α or TNFR1 were found to be resistant to endotoxic shock, further emphasizing a central role for TNF α. Although, anti-TNF therapy has not worked in patients with sepsis, this can be explained by the early rise in TNF prior to the institution of a specific therapy.

The liver has been traditionally thought to be a major source of TNFα production during the course of endotoxic shock. Despite the accepted central role of TNFα in endotoxic shock, the proinflammatory pathways that activate NFκB, and the consequences of this activation, remain quite complex and poorly understood. Our studies demonstrating the ability of a dominant IKKβ mutant, but not IKKα, to reduce NFκB activation in the liver and serum levels of TNFα under conditions of endotoxic shock are consistent with previous work in cell lines. Moreover, ectopic IKKβ/KA expression decreased LPS induced serum ALT levels and liver apoptosis, which appeared to correlate well with survival. This reduction of LPS induced liver apoptosis by IKKβ/KA was an interesting finding, since severe liver degeneration and enhanced apoptotic responses to TNFα have been observed in knockout mice and fibroblasts lacking IKKβ, respectively. Furthermore NEMO/IKKγ-deficient mice also demonstrate severe liver degeneration supporting the importance of NFκB in hepatocellular survival. Hence, NFκB functions as an anti-apoptotic factor during embryonic liver development and a functional IKKβ subunit is required for this process. However, results from our present study concerning NFκB involvement in hepatocellular apoptosis following LPS exposure, suggest that pro-inflammatory and anti-apoptotic functions of NFκB may be uniquely regulated in the adult and fetal liver.

Two reasons might account for this observation. First, our approach did not completely inhibit NFκB activation (Figure 1C) leaving the potential for residual anti-apoptotic effects. A second possibility, as discussed above, may be that the post-natal hepatocellular dependence on NFκB for cell survival is different from that seen during the prenatal period. In support of this hypothesis, the expression of an inducible degradation resistant IκBα (S32A/S36A) transgene in adult mice did not lead to any liver dysfunction in the absence of infection over a 15 month period despite the inability of the liver to induce NFκB. Our findings demonstrating a lack of enhanced apoptosis in mice expressing IκBβ/KA in the liver is most consistent with results from this previous report. However, others have found that inhibition of NFκB with a dominant negative IκBα encoding adenovirus leads to massive apoptosis following partial hepatectomy in adult mice. Nonetheless, it is plausible that partial hepatectomy models more closely represent stem cell regeneration in the fetal liver. Our studies suggest that balancing NFκB pro-inflammatory and anti-apoptotic consequences in the liver is critical for organism survival. The ability of IκBβ/KA to tilt this balance in favor of cell survival by reducing NFκB activation and TNFα production is likely responsible for the observed prolonged survival. Furthermore, these studies suggest that the liver is a primary organ of influence in controlling the fatal outcome of endotoxic shock.

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