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Adenoviral Infection Decreases Mortality from Lipopolysaccharide-Induced Liver Failure Via Induction of TNF-α Tolerance

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Effects of adenoviral infection on in vivo responses to LPS mediated by TNF-α were evaluated in a murine model. Adenovirus-infected mice showed decreased mortality from fulminant hepatitis induced by administration of LPS or staphylococcal enterotoxin B in the presence of D-galactosamine. Importantly, TNF-α resistance genes within adenoviral E3 region were not required, because E1,E3-deleted vectors showed similar effects. Adenovirus-infected mice exhibited higher TNF-α levels after LPS stimulation, no difference in TNFR1 expression, and similar mortality from Fas-induced fulminant hepatitis. Decreased production of IL-6 and KC in response to exogenous TNF-α, in addition to protection from TNF-α, suggested that adenoviral infection results in TNF-α tolerance.

provides first evidence that TNF-α tolerance may be induced by a viral infection.

Materials and Methods

Adenoviral infection

Serotype 5 human adenovirus was purchased from American Type Culture Collection (ATCC, VR-5, Manassas, VA) or from Advanced Biotechnologies (Columbia, MD). Replication-deficient E13-deleted adenoviral vectors expressing enhanced green fluorescence protein (Ad.GFP) or freely luciferase were obtained from the University of Iowa Gene Transfer Vector Core. The typical titers of the vectors were in the range of 1.2 × 10^6 to 2 × 10^10 PFU/ml, as determined by spectrophotometry or by plaque assay on 293 cells, respectively. C57BL/6 or DBA/2, 6- to 8-wk-old female mice were purchased from Harlan (Indianapolis, IN). Adenoviral infections were established by i.v. injections of the viruses to ketamine/xylazine-anesthetized mice (8.9 × 10^6 PFU/mouse of the wild-type and 10^5-10^6 PFU/mouse of the vectors in 100 μl vol). Sterile carrier solution (3% sucrose in PBS) was used for control injections and for dilution of the viruses. Control mice were anesthetized and received 100 μl of the carrier solution, and were usually housed in the same biohazard containment rooms as the adenovirus-infected mice.

Fulminant hepatitis models

Mice were sensitized by i.p. administration of 25 mg/mouse of D-Gal (Sigma-Aldrich, St. Louis, MO). LPS from Escherichia coli 0111:B4 (Sigma-Aldrich) was used at 50 μg/kg. Staphylococcal enterotoxin B (Toxin Technology, Sarasota, FL) was used at 2.5 mg/kg. Mouse rTNF-α (Pierce Biotechnology, Rockford, IL) with sp. act. 10^11 IU/μg (based on comparison with mouse TNF-α National Institute for Biological Standards and Control Standard) and endotoxin content 0.019 EU/g was used at 0.1 μg/mouse. All reagents were diluted in sterile PBS and used immediately after sensitization with D-Gal. No azide/low endotoxin hamster anti-mouse Fas mAb Jo-2 (BD Biosciences, San Jose, CA) was used at 10 μg/mouse. Mice were killed for up to 28 h after LPS or anti-Fas treatments and for up to 72 h after SEB or TNF-α treatments with periodic observations every 1 h during initial 12 h and every 4 h thereafter. The animal protocols were approved by the University of Iowa Institutional Animal Care and Use Committee.

Analysis of liver injury

Control or Ad.GFP-infected mice were euthanized before or 6 h after treatment with a lethal dose of LPS/D-Gal. Liver lobes were excised and fixed by submersion in 4% paraformaldehyde in PBS for 2 h. Cryosections were prepared following cryoprotection in 20% sucrose and embedding in Tissue-Tek OCT compound (Sakura Finetek USA, Torrance, CA) and used for TUNEL assay for apoptosis or H&E staining immediately after sensitization with D-Gal. No azide/low endotoxin hamster anti-mouse Fas mAb Jo-2 (BD Biosciences, San Jose, CA) was used at 10 μg/mouse. Mortality was evaluated for up to 28 h after LPS or anti-Fas treatments and for up to 72 h after SEB or TNF-α treatments with periodic observations every 1 h during initial 12 h and every 4 h thereafter. The animal protocols were approved by the University of Iowa Institutional Animal Care and Use Committee.

Adenoviral DNA isolation and PCR

Adenoviral DNA was isolated from 10^10 particles of the wild-type adenovirus (Advanced Biotechnologies) or Ad.GFP DNA using Easy DNA kit (Invitrogen, Carlsbad, CA) and measured using PicoGreen dsDNA quantitation kit (Molecular Probes, Eugene, OR), following manufacturer’s recommendations. The amplifications were conducted using 4 ng of the adenoviral DNA as template and 2.5 U of platinum Taq polymerase (Molecular Probes, Eugene, OR) following manufacturer’s recommendations. The amplification kit and sequenced at the DNA Facility. Enzyme immunoassays and Western blotting

Serum and liver samples were obtained from euthanized mice at different times after treatments. Serial dilutions of the serum were assayed for mouse TNF-α, soluble TNFR1, IL-6, and KC using DuoSet ELISA development kits (R&D Systems, Minneapolis, MN), following manufacturer’s recommendations. Livers were immediately frozen in liquid nitrogen and stored at −70°C until they were homogenized in 3 vol of ice-cold lysing buffer (0.05 M Tris, pH 7.4, 0.15M NaCl, 1% Nonidet P-40) supplemented with protease inhibitors (Roche Molecular Biochemicals) and 1× phosphatase inhibitors (Calbiochem, La Jolla, CA). The homogenates were centrifuged twice at 16,000 × g at 4°C for 10 min, and supernatants were assayed for total protein content using a Bio-Rad protein assay kit and for TNFRI content using the DuoSet kit for mouse soluble TNFR1. The amounts of TNF-α bound to TNFR1 were measured using the capture Ab against mouse TNFR1 and biotinylated Ab against mouse TNF-α from the corresponding DuoSet kits. Standard curve was generated using the capture Ab against TNF-α, mouse rTNF-α, and biotinylated Ab against TNF-α. The data were normalized to protein concentrations in the samples.

Western blotting analysis was performed by separating 40 μg of liver lysates in 10% SDS-PAGE gel and semi-dry transfer onto nitrocellulose membrane (Amersham, Arlington Heights, IL). The membranes were blocked with 5% milk in TTBS (TBS with 0.1% Tween 20) for 1 h and incubated with the rabbit anti-mouse TNFR1 Ab (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. The blots were washed four times with TTBS and incubated for 1 h with HRP-conjugated secondary Abs. Immunoreactive bands were developed using a chemiluminescent substrate (ECL Plus; Amersham). Autoradiographs were obtained by exposing Kodak Biomax MR films (Eastman Kodak, Rochester, NY) to the membranes for 10 s to 5 min. The intensity of the immunoblotting signals was measured using Fluor-S Multimager system and Quantity One software (Bio-Rad).

TNF-α bioassay

L929 cells (ATCC, CCL-1) were plated overnight at 4 × 10^4 cells/well in 96-well plates and incubated with serial dilutions of the serum samples in the presence of 2.5 μg/ml actinomycin D (Calbiochem) for 16 h. Mouse rTNF-α (Pierce) with sp. act. 10^11 IU/μg (based on comparison with mouse TNF-α National Institute for Biological Standards and Control Standard) was used to generate the standard curves. Cell viability was evaluated by spectrophotometric measurement of reduction of water-soluble tetrazolium salt (Roche Molecular Biochemicals).

Statistical analyses

Survival curves between control and adenovirus-infected mice were compared using χ² log rank tests. Continuous results were expressed as means ± SEM and analyzed using one-way ANOVA test. If a significant difference was found (p < 0.05), the individual groups were compared using Bonferroni postanalysis test. All calculations were performed with GraphPad Prism software version 3.0 (GraphPad Software, San Diego, CA).

Results and Discussion

Adenoviral infection decreases LPS-induced mortality

Adenoviral infection in the C57BL/6 mice was established by i.v. injection using serotype 5 human adenovirus 2, 7, and 14 days before challenge with LPS/D-Gal. Control and adenovirus-infected mice were injected i.p. with D-Gal and LPS and monitored for survival for up to 28 h. Mortality rate in the control group was consistent with previous studies (24–26). Mice that had been infected with adenovirus for 7 days showed significantly decreased mortality rate: the first death was recorded at 12 h and 50% mice survived after 28 h (Fig. 1). Mice in all other groups died within 15 h. Adenoviral infection 2 days before LPS challenge showed slightly delayed mortality, whereas the mice that had been infected for 14 days showed mortality rate indistinguishable from the control mice. The protection at 7 days postadenoviral infection was statistically significant, as confirmed by two additional independent experiments with two groups; all control mice died within 9 h
after LPS challenge; at least 50% adeno virus-infected mice survived LPS challenge and showed no signs of sickness after 28 h (p < 0.01; data not shown). Thus, systemic infection with wild-type adenovirus transiently decreases LPS-induced mortality.

TNF-α, released by activated macrophages, dendritic cells, and Kupffer cells, has been shown to be the key mediator of liver injury in the LPS model inducing massive apoptosis in the liver (24–26, 39). Adenoviral proteins E1B-19K, E3–14.7K, E3–6.7K are known to protect infected cells from TNF-α-induced apoptosis (21). Expression of E1B-19K protein is not likely to play any role in our system because it has been shown to protect human cells, but not mouse cells (40). However, constitutive overexpression of E3–14.7K protein has been shown to protect mice from LPS-induced acute liver failure (23). To determine whether adenoviral TNF resistance genes within E3 region mediate protection from LPS in our model, we used recombinant replication-deficient adenoviral vectors that were generated using the backbone of previously described adenoviral proteins (41). In addition, this vector is rendered replication deficient by a deletion (Ad5 bp 354–10.4K, E3–14.7K, and E3–14.4K adenoviral proteins (41). In addition, this vector is rendered replication deficient by a deletion (Ad5 bp 354–10.4K, E3–14.7K, and E3–14.4K adenoviral proteins (41). 

Adenoviral infection was established in C57BL/6 mice by i.v. injection of 109 PFU/mouse of replication-deficient E1- and E3-deleted Ad.GFP. Control mice were injected with equivalent volume of the carrier (3% sucrose in PBS). LPS and D-Gal were administered on day 7 postinfection. Mortality rate was significantly different (p < 0.005; n = 4 mice per group). Ad.GFP vector has dl309 deletion and no contaminating wild-type adenovirus. DNA was isolated from wild-type adenovirus (lanes 2 and 4) and Ad.GFP (lanes 3 and 5) and amplified using primers specific for human serotype 5 adenovirus DNA (lanes 2 and 3) or primers flanking previously described dl309 deletion/insertion (lanes 4 and 5). Lane 1, 100-bp DNA ladder.

Adenovirus-infected mice have higher TNF-α levels and bioactivity following LPS stimulation

We have previously reported that adenovirus-infected mice had lower levels of serum TNF-α late in sepsis induced by cecal ligation and puncture (12). Decreased production of TNF-α following LPS stimulation could potentially explain the differences in the mortality rate between control and Ad.GFP-infected mice. No TNF-α was detected in the sera of control or Ad.GFP-infected mice before LPS stimulation (sensitivity of the assay, 8 pg/ml; data not shown). However, we found significantly higher serum TNF-α levels in the Ad.GFP-infected mice relative to control mice at 90 min following LPS treatment, when the levels of TNF-α have been reported to peak (39) (Fig. 3A). No difference in the TNF-α levels was found at 3 h. Thus, adenovirus-infected mice exhibit higher levels of TNF-α in the circulation, yet remain resistant to its hepatotoxic effects.

Soluble TNF receptors stoichiometrically bind and regulate availability and biological activity of TNF-α in the circulation (42). Soluble rTNFR1 administered i.v. or overexpressed with an adenoviral vector is capable of protecting mice from LPS (43, 44).
TNF-α and a number of other LPS-inducible mediators (IL-6, IL-1β, etc.) are known to induce release of soluble TNFRs via ectodomain shedding (45–47). Adenoviral infection resulted in significant increase of serum soluble TNFR1 levels (2276 ± 516 pg/ml in control mice vs 5136 ± 628 pg/ml in Ad.GFP-infected mice on day 7 postinfection, p < 0.05). We have previously reported that Ad.GFP-infected mice have dramatically higher serum levels of soluble TNFR1 than the control mice at 24 h following cecal ligation and puncture (12). For these reasons, we measured soluble TNFR1 in the serum at 90 min and 3 h following stimulation with LPS to determine whether increased shedding of soluble TNFR1 occurs in this model. The levels of soluble TNFR1 were dramatically higher at 90 min after LPS treatment and returned to the basal levels by 3 h in both groups. Ad.GFP-infected mice had significantly higher levels of soluble TNFR1 than the control mice at both time points (Fig. 3B). These data suggest that adenoviral infection primes for increased TNFR1 shedding following LPS stimulation.

The in vivo interactions between soluble TNFRs and TNF-α are complex; soluble TNFRs may serve as TNF-α antagonists when present in large excess, as TNF carrier proteins, or as stabilizers of TNF-α bioactivity (42). Moreover, additional serum factors, such as soluble TNFR2 or α2-macroglobulin, may also regulate TNF-α bioactivity. To determine the amount of biologically active TNF-α in the serum of control and adenovirus-infected mice following LPS stimulation, we performed a bioassay using TNF-α-sensitive L929 cells pretreated with actinomycin D. We found that serum collected from Ad.GFP-infected mice 90 min after LPS treatment was significantly more toxic to L929 cells than the serum from control mice (Fig. 3C). Thus, adenovirus-infected mice have higher levels of biologically active TNF-α, despite the presence of increased soluble TNFR1. Therefore, increased levels of soluble TNFR1 are not sufficient to block the systemic effects of TNF-α in our system. These data also suggest that protection of adenovirus-infected mice from LPS/D-Gal-induced liver failure cannot be explained by the presence of any other TNF-α-inhibiting factors in the serum.

**Figure 4.** Adenoviral infection protects from liver failure induced by SEB/D-Gal. DBA/2 mice were infected with Ad.GFP for 7 days and challenged with 2.5 mg/kg SEB and 25 mg/mouse D-Gal. Mortality rate was significantly different (p < 0.01; n = 4 mice per group).
Adenoviral infection has no effect on expression of TNFR1 in the liver

Adenoviral proteins E3–10.4K and E3–14.5K form a receptor internalization and degradation complex that protects cells from apoptosis induced by signaling through Fas (52). This complex has been shown to associate with E3–6.7K protein and internalize two other death receptors (TNF-related apoptosis-inducing ligand-1 and TNF-related apoptosis-inducing ligand-2) belonging to the TNFR family (53). TNFR1 has been shown to be necessary and sufficient to mediate proapoptotic TNF-α signaling in LPS- or TNF-α-mediated fulminant hepatitis with D-Gal sensitization (25, 54). Adenoviruses are not known to decrease TNFR1 expression.

However, elevated levels of soluble TNFR1 indicate increased shedding of TNFR1 in response to LPS. We evaluated expression of TNFR1 using immunoblotting analysis to exclude the possibility that adenovirus-induced tolerance to TNF-α may be mediated by decreased expression of TNFR1. No difference was found in the levels of TNFR1 between control and Ad.GFP-infected mice even after LPS treatment (Fig. 6A). Furthermore, we used an ELISA to measure TNFR1 contents in the pooled liver lysates obtained from mice following Ad.GFP infection and LPS treatment (Fig. 6B). We found that TNFR1 expression in the liver decreased following LPS treatment in both groups; however, no difference was evident between control and Ad.GFP-infected mice. These data suggest that adenoviral infection does not affect TNFR1 expression in the liver.

An assay for TNF-α bound to the liver TNFR1 following LPS treatment has also revealed no significant difference between control and Ad.GFP-infected groups (data not shown). This suggests that adenovirus-induced TNF-α tolerance manifests downstream of TNFR1 signaling.

**Adenoviral infection-induced TNF-α tolerance decreases liver injury following LPS treatment**

To determine whether decreased mortality from LPS-induced liver failure correlates with the degree of liver injury, we analyzed liver sections prepared from control and Ad.GFP-infected mice (day 7 postinfection) before and after 6-h treatment with lethal dose of LPS/D-Gal (Fig. 7A). No apoptosis was detected by TUNEL assay in previous studies using the models of LPS-induced fulminant hepatitis (26, 36). Few apoptotic nuclei were found in the liver sections from Ad.GFP-infected mice without treatment. Extensive apoptosis was found in the liver sections of mice treated with LPS/D-Gal, which is consistent with previous studies using the models of LPS-induced fulminant hepatitis (26, 36). Few apoptotic nuclei per field (Fig. 7B). H&E staining of liver sections were found between control and adenovirus-infected mice in the basal levels of IL-6 or KC (data not shown). We found that adenoviral infection resulted in decreased peak levels of IL-6 (Fig. 5B) and KC (Fig. 5C) after TNF-α treatment. These data suggest that adenoviral infection not only inhibits TNF-α-induced apoptosis in the liver, but also down-regulates proinflammatory responses to TNF-α.

**FIGURE 5.** Adenoviral infection results in TNF-α tolerance. A, Adenovirus-infected mice are protected from liver failure induced by TNF-α/D-Gal. C57BL/6 mice were injected with 8.9 × 10³ PFU/mouse of wild-type adenovirus (wtAd, n = 5), 10³ PFU/mouse of Ad.GFP (n = 6), or 100 μL 3% sucrose in PBS (control, n = 6) 7 days before challenge with 0.1 μg/mouse TNF and 25 mg/mouse D-Gal. B and C, Adenovirus-infected mice produce less IL-6 and KC in response to TNF-α. Serum IL-6 (B) and KC (C) were measured in control and wild-type adenovirus-infected mice following treatment with 4 μg/mouse of mouse TNF-α (without D-Gal). Three mice per group were euthanized at each time point to collect sera, and serum samples were processed individually. *, p < 0.05; **, p < 0.001.

**FIGURE 6.** Adenoviral infection has no effects on TNFR1 expression in the liver. A, TNFR1 expression in the livers from control and Ad.GFP-infected mice was evaluated by Western blotting at day 7 postinfection and 90 min after LPS/D-Gal treatment. Fifty micrograms of pooled liver lysates (4 mice per group) were separated on 10% SDS-PAGE and probed with rabbit polyclonal Ab against TNFR1. Densitometric analysis revealed no difference between the groups. B, ELISA was used to measure TNFR1 content in the pooled liver lysates (n = 4 mice per group) following LPS/D-Gal treatment. The data were normalized to protein concentrations in the samples.
revealed noticeable leukocyte infiltration in the livers of Ad.GFP-infected mice, which is not observed in normal livers (data not shown). Treatment of control mice with LPS/D-Gal resulted in extensive hemorrhage and necrosis in the livers typical for LPS-induced fulminant hepatitis (26, 36). The extent of liver hemorrhage and necrosis was substantially lower, albeit not eliminated in Ad.GFP-infected mice following LPS/D-Gal treatment (data not shown).

In summary, the livers of adenovirus-infected mice display leukocyte infiltration and a low level of apoptosis. Hemorrhage and increased apoptosis were evident in Ad.GFP-infected mice after LPS/D-Gal treatment, although their extent was significantly lower relative to LPS-treated control mice. Thus, adenoviral infection only partially decreases the extent of liver injury mediated by LPS-induced TNF-α; nevertheless, this may be sufficient to decrease mortality in this model.

Adenoviral infection does not protect from liver failure induced by Fas Ab

Adenoviruses are known to inhibit cellular apoptosis at several levels, including internalization of the death receptors, interactions with Bax and Bak, and inhibition of p53 (21). Agonistic Abs against Fas receptor are known to trigger apoptosis in the liver and fulminant hepatitis similarly to TNF-α (55). Proapoptotic signaling via TNFR1 and Fas receptor converges at the level of recruitment of procaspase-8 to Fas-associated death domain protein (56). To determine whether adenovirus-induced TNF-α tolerance is mediated by inhibition of apoptosis, we treated control and adenovirus-infected mice with the well-characterized Fas agonist Ab Jo-2 (Fig. 8). Control mice displayed 67% mortality following Jo-2 administration. Infection with the wild-type adenovirus slightly delayed mortality; none of these mice died within the first 8 h after the treatment, but all succumbed by 24 h. The mortality rate in the group of mice infected with Ad.GFP vector was similar to the control. Thus, it is not likely that inhibition of apoptosis explains protection from TNF-α following adenoviral infection.

In summary, our study shows that adenoviral infection primes for increased production of TNF-α and shedding of TNFR1 in response to LPS, but induces tolerance to hepatotoxic effects of TNF-α. This TNF-α tolerance protects mice from fulminant hepatitis triggered by LPS, SEB, or exogenous TNF-α. Another novel observation is that the effects of adenovirus are not dependent on previously described TNF-α resistance genes E1B-19K, E3–14.7K, E3–10.4K, and E3–14.5K, because E1- and E3-deleted adenoviral vector renders mice TNF-α tolerant. Importantly, adenovirus-infected mice were protected from LPS despite increased production and serum bioactivity of TNF-α following LPS stimulation. This demonstrates that adenoviral infection does not result in endotoxin tolerance, which is characterized by poor responses to LPS, such as decreased production of TNF-α (57). Furthermore, this separates adenovirus from influenza virus and lymphocytic choriomeningitis virus, which have been previously shown to increase mortality from LPS- and/or SEB-mediated shock (2–5).

It is well established that excessive TNF-α signaling may be harmful by inducing shock (58). Overexpression of soluble TNFR1 or TNFR1 deletion in transgenic mice protects against lethal effects of LPS (14, 15, 44). In contrast, TNF-α has been shown to be necessary for antibacterial host response because defects in TNF-α production or TNFR1 signaling lead to increased sensitivity to bacterial infections (14–17, 44). Our previous study demonstrated that adenovirus-infected mice have higher mortality from sepsis induced by cecal ligation and puncture (12). The fact that adenovirus-infected mice had significantly higher bacterial
load in the blood, liver, and lungs in that study suggests that antibacterial host response was impaired. This is further supported by our present data showing decreased production of IL-6 and KC in adenovirus-infected mice following TNF-α administration. We believe that adenovirus-induced TNF-α tolerance may contribute to the lethal synergy between adenoviral infection and sepsis, although a number of additional mechanisms may be implicated. It remains to be determined whether adenovirus-induced TNF-α tolerance inhibits protective functions of TNF-α during bacterial infections and sepsis.

Further studies will be necessary to precisely determine the mechanism of adenovirus-induced TNF-α tolerance. A number of instances and mechanisms of TNF-α tolerance have been reported previously. Low doses of TNF-α, IL-1, or LPS; exposure to heat shock; or genetic defects may render mice TNF-α-sensitive. Additional mechanisms may include transcriptional signaling downstream of Fas-associated death domain protein, shared by Fas receptor, appears to be intact in adenovirus-infected mice. It is also not likely that protection is mediated by NF-κB-mediated antiapoptotic signaling via TNFR1 or TNFR2, because D-Gal sensitization prevents gene transcription in liver cells (24, 25). It is reasonable to suggest that adenovirus-induced TNF-α tolerance leads to a defect in the proximal TNFR1 signaling.

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References

FIGURE 8. Adenoviral infection does not protect from Fas-induced liver failure. C57BL/6 mice were infected with 8.9 × 10^5 PFU/mouse of wild-type adenovirus (wtAd), 10^6 PFU/mouse of Ad.GFP, or 100 μl 3% sucrose in PBS (control) 7 days before challenge with 10 μg/mouse of anti-Fas Ab Jo-2 (n = 6 mice per group).


