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RESEARCH PAPER

Antileukoproteinase protects against hepatic inflammation, but not apoptosis in the response of D-galactosamine-sensitized mice to lipopolysaccharide

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Background and purpose: There is major evidence for the strong bi-directional interrelation of parenchymal cell apoptosis and leukocyte accumulation and inflammation in acute liver injury. Therefore, the aim of this *in vivo* study was to investigate the anti-apoptotic and anti-inflammatory potential of antileukoproteinase (ALP) in a murine model of acute liver failure.
Experimental approach: C57BL/6J mice were given galactosamine (D-GalN) and *E. coli* lipopolysaccharide (LPS) followed by administration of saline or ALP. Besides survival rate, hepatic tissue damage and inflammatory response were analyzed by intravital fluorescence microscopy 6 hours after treatment. In addition, immunohistochemical analysis of NFκB-p65 and hepatocellular apoptosis, plasma levels of AST/ALT, TNF-α and IL-10 were determined.
Key results: Administration of D-GalN/LPS provoked hepatic damage, including marked leukocyte recruitment and microvascular perfusion failure, as well as hepatocellular apoptosis and enzyme release. NFκB-p65 became increasingly detectable in hepatocellular nuclei, accompanied by a rise of TNF-α and IL-10 plasma levels. ALP markedly reduced intrahepatic leukocyte accumulation, nuclear translocation of NFκB and plasma levels of TNF-α and IL-10. Moreover, liver enzyme levels indicated the absence of necrotic parenchymal cell death. In contrast, ALP failed to block both apoptosis and caspase-3 levels and the mortality rate of ALP-treated animals was comparable to that of saline-treated mice.
Conclusions and implications: ALP could effectively prevent D-GalN/LPS-associated intrahepatic inflammatory responses by inhibition of NFκB activity, but not apoptosis-driven mortality. Thus, a protease-inactivating approach such as application of ALP seems to be inadequate in damaged liver where apoptosis represents the predominant mode of cell death.
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Keywords: rodent; neutrophils; apoptosis; inflammation; liver; microcirculation

Abbreviations: ALP, antileukoproteinase; ALT, alanine aminotransferase; ANOVA, analysis of variance; AST, aspartate aminotransferase; CCD, charged coupled device; D-GalN, D-galactosamine; ECL, enhanced chemiluminescence; EDTA, ethylene diamine tetraacetic acid; IAP, inhibitor of apoptosis; Ig, immunoglobulin; IL, interleukin; i.p., intraperitoneal; i.v., intravenous; LPS, lipopolysaccharide; NFκB-p65, nuclear factor kappaB-phospho65; PE, polyethylene; PMSF, phenylmethane sulfonylfluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; s.e.m., standard error of the means; TNF-α, tumor necrosis factor alpha; TUNEL, terminal transferase dUTP nick end labelling

Introduction

Sepsis and endotoxemia are frequently involved in the development of fulminant hepatic failure, which still has a poor prognosis and leads to high mortality due to the lack of effective therapeutic strategies (Ostapowicz *et al*., 2002; Schiodt and Lee, 2003). Endotoxin, a Gram-negative bacterial lipopolysaccharide (LPS), is considered to contribute significantly to hepatic failure and multiple organ failure by the release of proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) (Bohlinger *et al*., 1996). Beside the various effects of TNF-α on leukocytes and liver cells that support inflammation, TNF-α can also induce apoptosis through the death domain of the TNF-receptor 1 (Tartaglia

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et al., 1993). Hepatocellular apoptosis not only represents the cardinal feature in acute liver failure, but also functions as a signal for leukocyte migration and attack on parenchymal cells (Jaeschke *et al*., 1998; Eipel *et al*., 2004), thereby establishing a vicious circle with aggravation of leukocytic inflammation and finally cell death. Leukocyte-dependent serine proteases, that is, neutrophil elastase and cathepsin, have been reported to cause considerable tissue damage (Jochum *et al*., 1994; Jaeschke and Smith, 1997), whereas blockade of these proteolytic enzymes resulted in a significant attenuation of parenchymal tissue injury (Welbourn *et al*., 1991; Moraes *et al*., 2006).

As a common experimental approach to study acute hepatic failure, mice are treated with a combination of LPS and D-galactosamine (D-GalN) representing a model of liver injury secondary to induction of potent apoptotic signals in the presence of transcriptional inhibition. Indeed, it has been shown that LPS toxicity in D-GalN-sensitized mice was due to TNF-α release and that LPS accounted for TNF-mediated hepatocellular apoptosis (Freudenberg and Galanos, 1991; Leist *et al*., 1995). A recent study further addressed the elementary step of nuclear factor kappaB (NFκB) shuttling in the temporal and spatial relation to hepatocellular apoptosis upon TNF-α exposure of D-GalN-sensitized mice (Tapalaga *et al*., 2002).

Antileukoproteinase (ALP), which is well known in its function as an inhibitor of serine proteases, modifies immune functions in mice such that ALP suppresses the macrophage response and increases the resistance to LPS (Taggart *et al*., 2002). This modulating effect is supposed to be due to interference of ALP with the NFκB pathway, as ALP prevents by an unknown mechanism the breakdown of IκBβ, an important cytosolic inhibitor of NFκB (Taggart *et al*., 2002; Ward and Lentsch, 2002). These considerations prompted us to study the efficacy of ALP in the prevention of hepatic injury and lethality upon exposure of mice to LPS and D-GalN.

Methods

Animal model and experimental groups

Female C57BL/6J mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and were used at 8–10 weeks of age with a body weight of approximately 20 g. Animals were kept on water and standard laboratory chow *ad libitum*. The experiments were conducted in accordance with the German legislation on protection of animals and the NIH Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council).

For induction of acute liver injury, animals were injected with D-GalN (720 mg kg^{-1} intraperitoneal (i.p.); Sigma-Aldrich, Taufkirchen, Germany) and LPS (10 μg kg^{-1} i.p.; serotype 0128:B12, Sigma-Aldrich) and received either saline or ALP (15 mg kg^{-1} i.p.). Additional animals received isotonic saline only. Concentrations of D-GalN and LPS were used in accordance with previously published work (Leist *et al*., 1995; Morikawa *et al*., 1996; Klintman *et al*., 2004).

Recombinant ALP

Human ALP was produced as a recombinant protein in *Escherichia coli* and subsequently purified to homogeneity by a multistep purification protocol using ion-exchange, metal-chelate and size-exclusion chromatography, originally described by Heinzel-Wieland *et al*. (1991). Analytical reverse-phase chromatography revealed a single peak and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) exhibited a single band of the expected electrophoretic mobility upon silver staining. The material was tested for inhibition of granulocyte elastase and cathepsin G. To ensure the removal of any endotoxin contamination, the ALP charges were run on detoxi-gel columns (polymyxin B-conjugated columns; Perbio Science, Bonn, Germany) following the manufacturer's instructions. Routine testing of the final ALP preparations before use showed, for all analyzed samples, that the endotoxin content remained below the detection limit (0.05 EU ml^{-1}) of the applied *Limulus* amoebocyte lysate gel-clot assay (Sigma-Aldrich).

Intravital fluorescence microscopy

Hepatic microcirculation was assessed in mice ($n=6$ per group) at 6 h after pretreatment with drugs. Anesthetized animals (ketamine 75 mg kg^{-1} i.p. and xylazine 25 mg kg^{-1} i.p.) were placed in the supine position on a heating pad for maintenance of body temperature at 36–37°C. Insertion of a polyethylene catheter (PE 50, ID 0.58 mm, Portex, Hythe, UK) in the right jugular vein served for injection of fluorescent dyes. After transverse laparotomy, the animals were positioned on their left side and the left liver lobe was exteriorized and covered with a glass slide for intravital fluorescence microscopy. Using a Zeiss fluorescence microscope with a 100 W mercury lamp and different filter sets for blue, green and ultraviolet epi-illumination (Axiotech vario, Zeiss, Jena, Germany), microscopic images were taken by a water immersion objective ($\times$20 0.50 W; Zeiss), televised using a CCD video camera (FK 6990A-IQ, Pieper, Berlin, Germany), and recorded on videotape for subsequent off-line evaluation (Eipel *et al*., 2004, 2005).

Blood perfusion within individual sinusoids was studied after tissue contrast enhancement by sodium fluorescein (2 μmol kg^{-1} intravenous (i.v.); Merck, Darmstadt, Germany) and blue light epi-illumination (450–490/$>$520 nm, excitation/emission wavelength) (Eipel *et al*., 2004). *In vivo* labelling of leukocytes with rhodamine-6G (1 μmol kg^{-1} i.v.; Merck) and green light epi-illumination (530–560/$>$580 nm) enabled quantitative analysis of leukocyte flow behavior in both sinusoids and postsinusoidal venules (Eipel *et al*., 2004). Assessment of hepatic microcirculatory parameters was performed off-line by frame-to-frame analysis of the videotaped images at a magnification of 424-fold, using a computer-assisted image analysis system with a 19″ monitor (CapImage; Zeintl, Heidelberg, Germany). Within 10 acini per animal, microcirculatory analysis included the determination of the number of perfused sinusoids, given as the percentage of all sinusoids crossing a 200 μm raster line. Leukocyte–endothelial cell interactions were analyzed within 10 hepatic acini and 10 postsinusoidal venules per animal, including (i) the number of stagnant leukocytes, located

within sinusoids (given as cells mm^{-2} observation field) and not moving during an observation period of 20 s, and (ii) the number of adherent leukocytes, located within postsinusoidal venules (given as cells mm^{-2} endothelial surface, calculated from the diameter and length of the vessel segment studied, assuming cylindrical geometry), and not moving or detaching from the endothelial lining during an observation period of 20 s (Eipel *et al*., 2004; Abshagen *et al*., 2006).

Sampling and assays

A set of additional animals was injected with D-GalN/LPS with or without application of ALP as described above. At 4 or 6 h after induction of liver injury, animals were exsanguinated by retro-orbital puncture under ketamine/xylazine anesthesia and blood as well as liver tissue was sampled for subsequent analysis ($n=5$–6 per group and time point). Animals receiving saline alone served as controls ($n=6$ per time point). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) plasma activities were measured spectrophotometrically as indicators for hepatocellular disintegration and necrosis.

Bioplex protein array system

A panel of plasma cytokines was measured in duplicate using the Bioplex Protein Array System (Bio-Rad Laboratories GmbH, Munich, Germany), according to the instructions of the manufacturer. This novel multiplexed, particle-based, flow cytometric assay, which utilizes anti-cytokine monoclonal antibodies linked to microspheres incorporating distinct proportions of two fluorescent dyes, enables quantification of several mediators in a sample volume as small as 12.5 μl. With the Bioplex cytokine assay kit in combination with the Bioplex Manager software we detected and quantified plasma TNF-α, interleukin (IL)-5 and IL-10. For each cytokine, eight standards ranging from 1.5 to 32 000 pg ml^{-1} were used.

Histology and immunohistochemistry

Liver tissue was fixed in 4% phosphate-buffered formalin for 2–3 days and then embedded in paraffin. From the paraffin-embedded tissue blocks, 5 μm sections were put on poly-L-lysine glass slides and exposed to an apoptosis-specific staining kit (indirect *in situ* DNA nick end labelling (TUNEL) assay; ApopTag, Chemicon International Inc., Temecula, CA, USA) according to the manufacturer's instructions. TUNEL-positive hepatocytes as well as TUNEL-positive non-parenchymal cells were counted within 30 consecutive fields ($\times 40$ objective, 0.65 numeric aperture) and given as positive events per mm^2.

For immunohistochemical analysis, liver tissue specimens on poly-L-lysine covered glass slides were exposed to a mouse monoclonal anti-human NFκB-p65 subunit antibody (1:300; Chemicon International, Temecula, CA, USA) followed by a secondary sheep anti-mouse Ig antibody (1:5000; Amersham Pharmacia Biotech, Freiburg, Germany). NFκB-p65-positive cells were counted within 30 consecutive fields and are given as positive hepatocellular nuclei per mm^2.

Western blot analysis

For Western blot analysis of cleaved caspase 3, liver tissue was homogenized in lysis buffer (10 mM Tris pH 7.5, 10 mM NaCl, 0.1 mM EDTA, 0.5% Triton X-100, 0.02% NaN_3, 0.2 mM phenylmethane sulfonylfluoride (PMSF), incubated for 30 min on ice and centrifuged for 15 min at 10 000 *g*. The supernatant was saved as a whole protein fraction. Before use, the buffer received a protease inhibitor cocktail (1:100 v/v; Sigma-Aldrich). Protein concentrations were determined using the bicinchoninic acid protein assay (Sigma-Aldrich) with bovine serum albumin as standard. Proteins (40 μg protein $lane^{-1}$) were separated discontinuously on sodium dodecyl sulphate polyacrylamide gels (12%) and transferred to a polyvinyldifluoride membrane (Immobilon-P, Millipore, Eschborn, Germany). After blockade of nonspecific binding sites, membranes were incubated for 2 h at room temperature with a rabbit anti-cleaved caspase-3 antibody (Asp 175; 1:1000; Cell Signaling Technology, Frankfurt, Germany) followed by a secondary peroxidase-conjugated goat anti-rabbit antibody (1:2000; Cell Signaling Technology). Protein expression was visualized by means of luminol enhanced chemiluminescence (ECL plus, Amersham Pharmacia Biotech) and exposure of the membrane to a blue-light-sensitive autoradiography film (Kodak BioMax Light Film, Kodak-Industrie, Chalon-sur-Saone, France). Signals were densitometrically assessed (Gel-Dokumentationssystem TotalLab, Nonlinear Dynamics, Newcastle upon Tyne, UK) and normalized to the β-actin signals (mouse monoclonal anti-β-actin antibody, 1:20 000; Sigma-Aldrich). Densitometric values of the respective protein levels in control mice were set as 1 and all other data are given as x-fold change.

Survival study

For assessment of survival, additional 30 animals underwent induction of acute liver failure (D-GalN 720 mg kg^{-1} i.p. and LPS 10 μg kg^{-1} i.p.) and were treated with either saline (100 μl), ALP (15 mg kg^{-1} i.p.) or both ALP and recombinant mouse interleukin (IL)-10 (1 μg $mouse^{-1}$ –10 min i.v.). Treatment with an IL-10-Fc fusion protein (Sigma-Aldrich) was performed in accordance with previously published work (Emmanuilidis *et al*., 2001; Manley *et al*., 2005). Mice ($n=10$ per group) that lived longer than 5 days were considered to be survivors.

Statistical analysis

All data are expressed as means $\pm$ s.e.m. After proving the assumption of normality and equal variance across groups, differences between groups were assessed using analysis of variance (ANOVA) followed by Dunn's method. Statistical significance was set at $P<0.05$. Statistics were performed using the software package SigmaStat (Jandel Corporation, San Rafael, CA, USA).

Results

Intravital fluorescence microscopy of the liver revealed characteristic features of acute liver failure in D-GalN/LPS-

treated mice, including intrahepatic white blood cell accumulation and sinusoidal perfusion failure. Besides massive intrahepatic leukocyte accumulation with increased numbers of adherent cells within the sinusoidal microvasculature (Figure 1a), D-GalN-LPS treatment significantly enhanced firm leukocyte adhesion in venules, as given by a seven-fold increase when compared to saline controls (Figure 1b). Further, D-GalN/LPS induced a hepatic damage characterized by an impairment of sinusoidal perfusion of about 25%, whereas in healthy controls almost all microvessels forwarded blood flow (Figure 1c). Of interest, ALP was able to reduce significantly the intrahepatic leukocyte accumulation, but without any noteworthy effect on nutritive perfusion (Figure 1). D-GalN/LPS-exposed liver tissue showed a marked induction of apoptotic cell death as indicated by a six-fold rise in cleaved caspase-3 protein expression (Figure 2) and a clear increase of both TUNEL-positive hepatocytes and non-parenchymal cells (Figure 3), without notable changes upon ALP treatment.

Plasma ALT and AST activities reflected the pronounced tissue damage particularly 6 h after D-GalN/LPS administration (Figure 4a and b). Interestingly, ALP treatment reduced enzyme release, almost to the values in saline-treated, control animals. However, ALP failed to prevent LPS-induced lethality in D-GalN-sensitized mice. After induction of liver failure with D-GalN/LPS, 8 out of 10 mice died within the

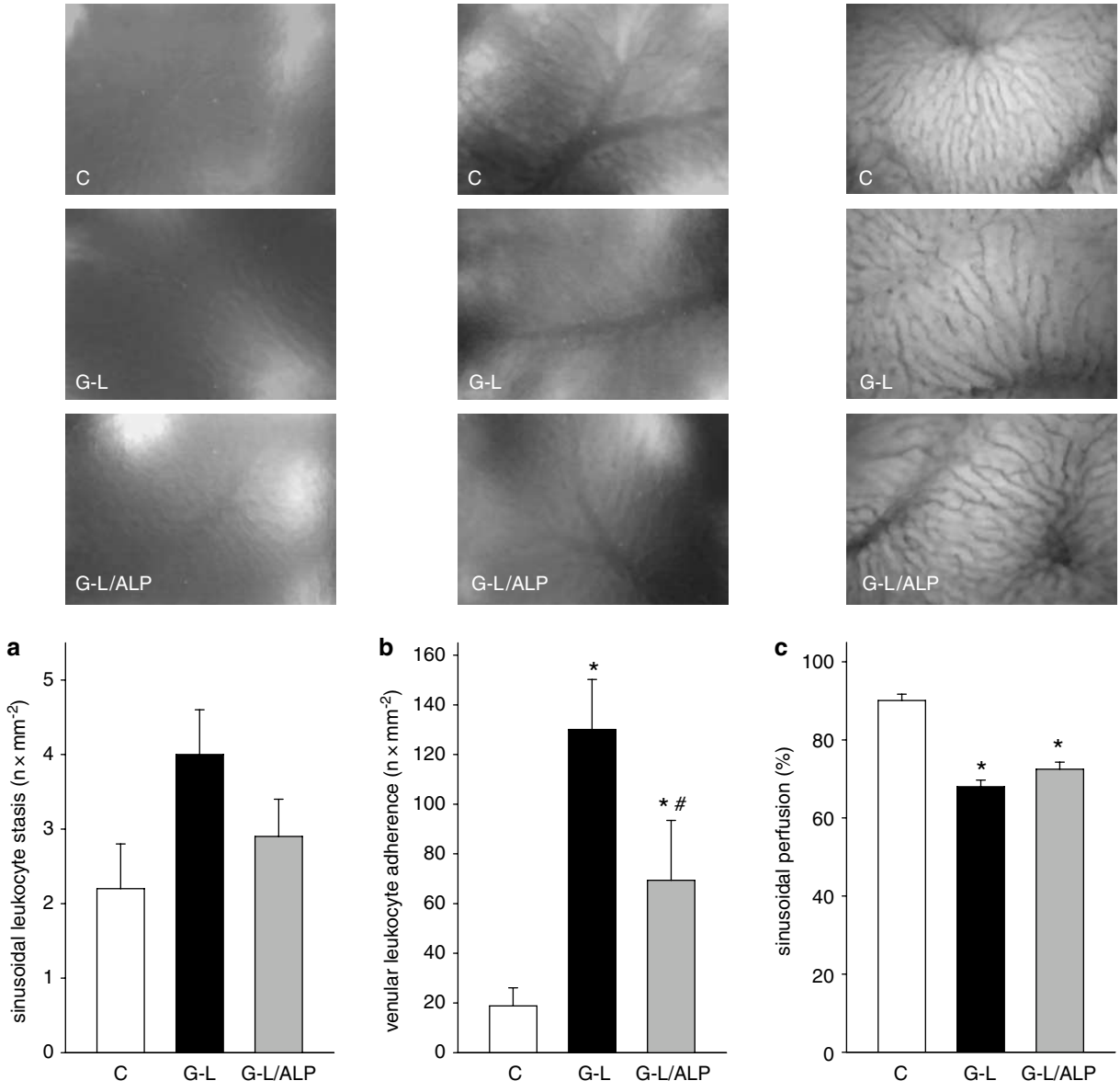

Figure 1 Representative intravital fluorescence microscopic images (upper panels, original magnification $\times 424$) of liver tissue after staining of leukocytes with rhodamine6G (**a, b**) and after contrast enhancement with sodium fluorescein (**c**) as well as quantitative analysis of sinusoidal leukocyte stasis (**a**), venular leukocyte adherence (**b**) and sinusoidal perfusion (**c**). For induction of acute liver injury, animals were injected with D-GalN (720 mg kg^{-1} i.p.) and LPS (10 μg kg^{-1} i.p.) and received either saline (G-L) or ALP (15 mg kg^{-1}; G-L/ALP). Control animals received isotonic saline only (C). Values are given as means $\pm$ s.e.m; ANOVA and Dunn's test; $^{*}P<0.05$ vs C; $^{\#}P<0.05$ vs G-L.

subsequent 7–10 h. Similarly, treatment with ALP was accompanied by a mortality rate of 90%. Combined application of ALP and IL-10 showed a slight tendency to delay death with a survival rate of 30% over this period (Figure 4c).

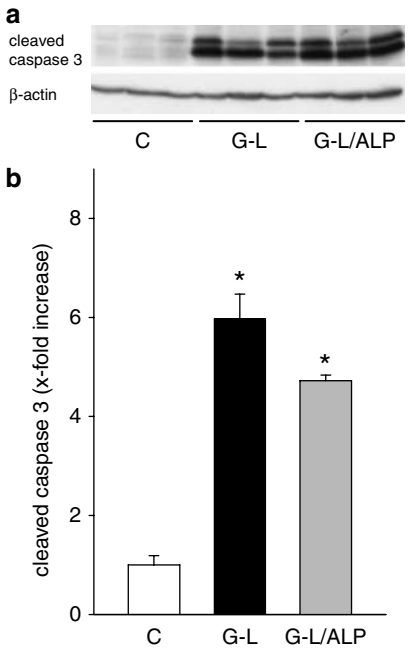

Figure 2 Representative Western blot analysis (**a**) and densitometry analysis (**b**) of cleaved caspase-3 protein expression. Signals were corrected with that of β-actin serving as internal control. For induction of acute liver injury, animals were injected with D-GalN (720 mg kg^{-1} i.p.) and LPS (10 μg kg^{-1} i.p.) and received either saline (G-L) or ALP (15 mg kg^{-1}, G-L/ALP). Control animals received isotonic saline only (C). Values are given as means$\pm$s.e.m.; ANOVA and Dunn's test; *$P<0.05$ vs C.

Immunohistochemistry of D-GalN/LPS-exposed liver tissue revealed an impressive activation of NFκB with predominant staining of hepatocellular nuclei (Figure 5b) when compared to controls (Figure 5a), while upon ALP treatment NFκB-p65 immunostaining was not only less pronounced but also markedly restricted to the cytoplasmic compartment of hepatocytes (Figure 5c). This nuclear exclusion of NFκB is best interpreted as inhibition of the nuclear shuttle of this transcription factor.

Plasma analyses revealed an increase of TNF-α and IL-10 concentrations upon D-GalN/LPS exposure, which were completely reversed by ALP treatment to values found in saline controls (Figure 6a and b). In contrast, IL-5 concentrations did not substantially differ between saline controls and D-GalN/LPS-exposed animals, whereas it was increased in mice upon ALP treatment (Figure 6c).

Discussion

In the present study, we demonstrate for the first time that ALP significantly attenuated D-GalN/LPS-associated intrahepatic inflammatory response and necrotic tissue injury, as indicated by the notable reduction of intrahepatic leukocyte recruitment and liver enzyme activities. Moreover, we could confirm that ALP interferes with the NFκB pathway by cytoplasmic restriction of this transcription factor and inhibition of NFκB-dependent downstream signals, such as TNF-α and IL-10. This is in accordance with comparable effects on cytokine-activated endothelial cells that have been described more recently by our group (Sehnert *et al.*, 2006).

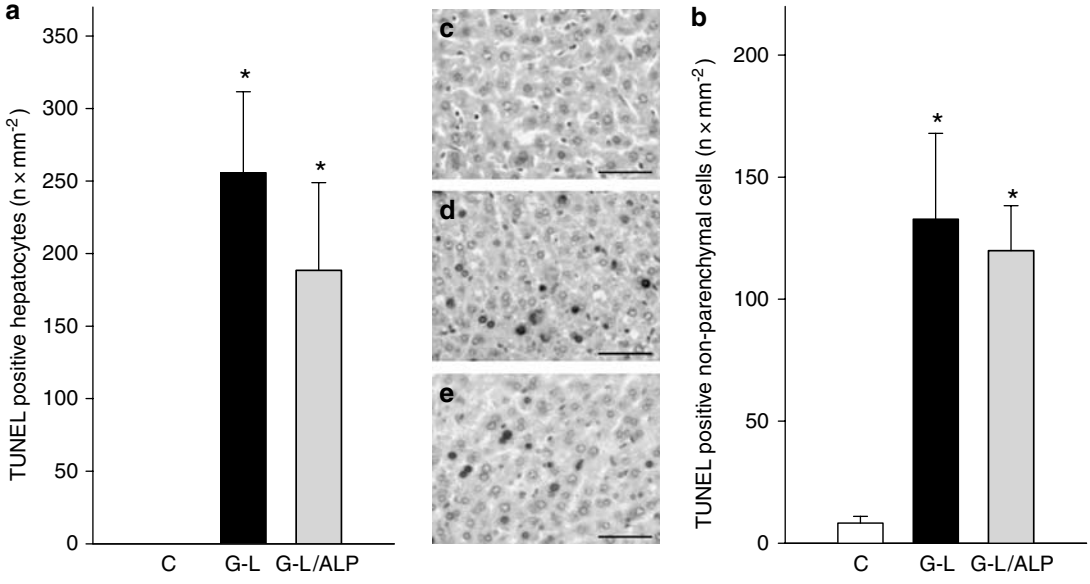

Figure 3 Quantitative analysis of TUNEL histochemistry, shown as number of TUNEL-positive cells per mm^2 differentiating between hepatocellular (**a**) and non-parenchymal cell apoptosis (**b**) as well as representative images of a saline- (**c**), G-L- (**d**) and G-L/ALP-treated animal (**e**). Bars represent 50 μm. For induction of acute liver injury, animals were injected with D-GalN (720 mg kg^{-1} i.p.) and LPS (10 μg kg^{-1} i.p.) and received either saline (G-L) or ALP (15 mg kg^{-1}; G-L/ALP). Control animals received isotonic saline only (C). Values are given as means$\pm$s.e.m.; ANOVA and Dunn's test; *$P<0.05$ vs C.

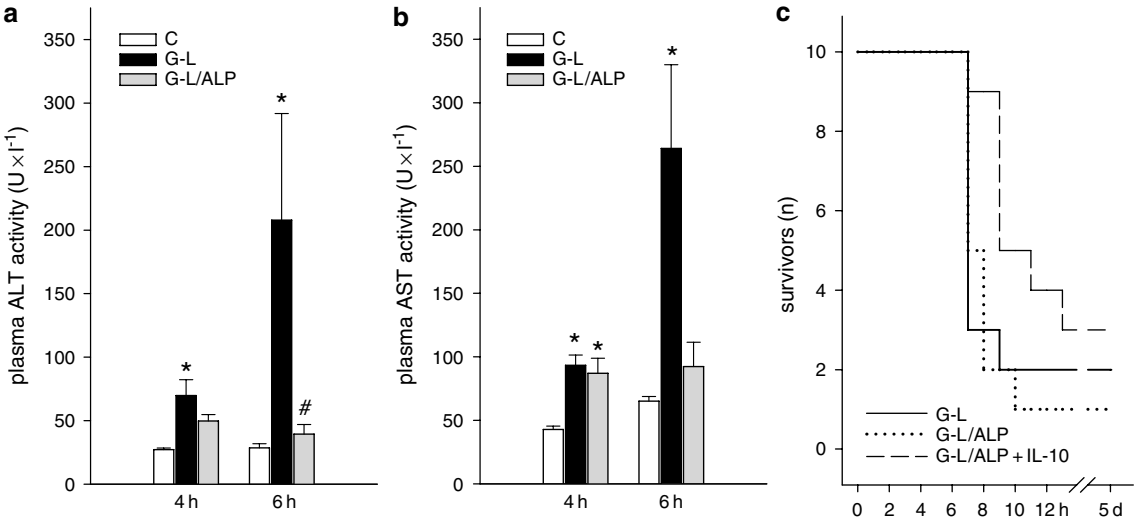

Figure 4 Plasma activities of ALT (**a**) and AST (**b**) at 4 and 6 h after induction of acute liver injury. Animals were injected with D-GalN (720 mg kg^{-1} i.p.) and LPS (10 μg kg^{-1} i.p.) and received either saline (G-L) or ALP (15 mg kg^{-1}; G-L/ALP). Control animals received isotonic saline only (C). Values are given as means ± s.e.m.; ANOVA and Dunn's test; *$P<0.05$ vs C. $^{\#}P<0.05$ vs G-L at the respective time points. For analysis of survival rate (**c**), mortality was assessed every hour during the first 24 h and every 12 h over a period of 5 days.

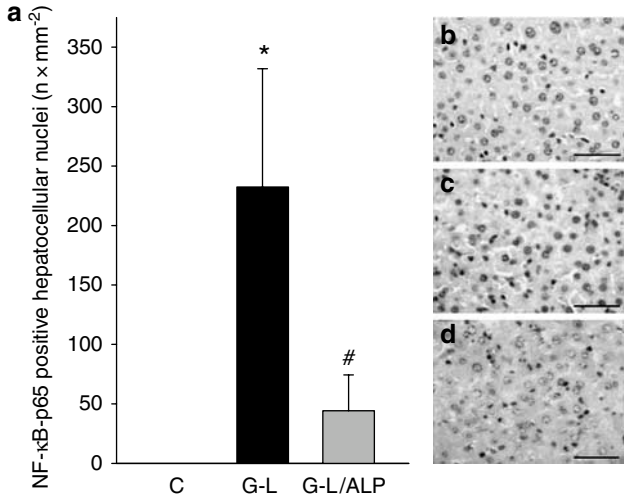

Figure 5 Quantitative analysis of NFκB-p65 immunohistochemistry, shown as number of NFκB-p65-positive hepatocytes per mm^2 (**a**), and representative images of a saline- (**b**), G-L- (**c**) and G-L/ALP-treated animal (**d**). Bars represent 50 μm. For induction of acute liver injury, animals were injected with D-GalN (720 mg kg^{-1} i.p.) and LPS (10 μg kg^{-1} i.p.) and received either saline (G-L) or ALP (15 mg kg^{-1}; G-L/ALP). Control animals received isotonic saline only (C). Values are given as means ± s.e.m.; ANOVA and Dunn's test; *$P<0.05$ vs C, $^{\#}P<0.05$ vs G-L.

The observation that exposure of animals to LPS or bacteria causes activation of NFκB in multiple organs and cell populations has led to the development of several approaches to inhibit nuclear translocation of NFκB in sepsis (Abraham, 2003; Zingarelli *et al.*, 2003). In line with this, suppression of NFκB activation has been shown to decrease acute inflammatory processes and organ dysfunction (Zingarelli *et al.*, 2003). The present study confirms this anti-inflammatory effect, as the primary parameters that are affected by ALP are those associated with NFκB-dependent inflammation. However, our results also clearly show that the benefits of ALP-induced suppression of NFκB activation did not improve overall lethality.

There is a body of evidence suggesting that suppression of NFκB function can also be harmful for the host (Sha *et al.*, 1995; Gjertsson *et al.*, 2001), as NFκB represents an essential component to mount a sufficient host defense to noxious stimuli. Thus, inhibition of NFκB with p65 antisense probes did not lead to beneficial effects in a staphylococcal-induced arthritis model with respect to mortality or severity of joint damage and bacterial burden was increased due to impaired clearance (Gjertsson *et al.*, 2001). Moreover, bacterial stimulation, either directly or through the non-parenchymal cells, could induce the hepatocytes to express NFκB-dependent chemokines, which are necessary for recruitment of bactericidal cells. Failure to induce the activity of the transcription factor would then compromise the ability of the organism to clear bacteria within the liver (Lavon *et al.*, 2000). Correspondingly, we could not observe a survival advantage of animals with the ALP-associated inhibition of NFκB. The role of NFκB to exert critical anti-inflammatory functions during the systemic response to LPS has further been attributed to its potential to inhibit several LPS-inducible proinflammatory genes (Gadjeva *et al.*, 2004) and to cause the transcription of anti-inflammatory cytokines, that is, IL-10 (Holmes *et al.*, 2003). In line with this, we observed a decrease in plasma IL-10 in ALP-treated mice. IL-10 is well recognized as an important negative regulator of proinflammatory genes induced in LPS-stimulated macrophages by modulating both the transcription and stability of specific mRNAs (DiPiro, 1997; Hamilton *et al.*, 2002). Experimental studies in rodents and primates have demonstrated that endogenously produced and exogenously administered IL-10 can reduce the magnitude of the inflammatory response and improve outcome, primarily in

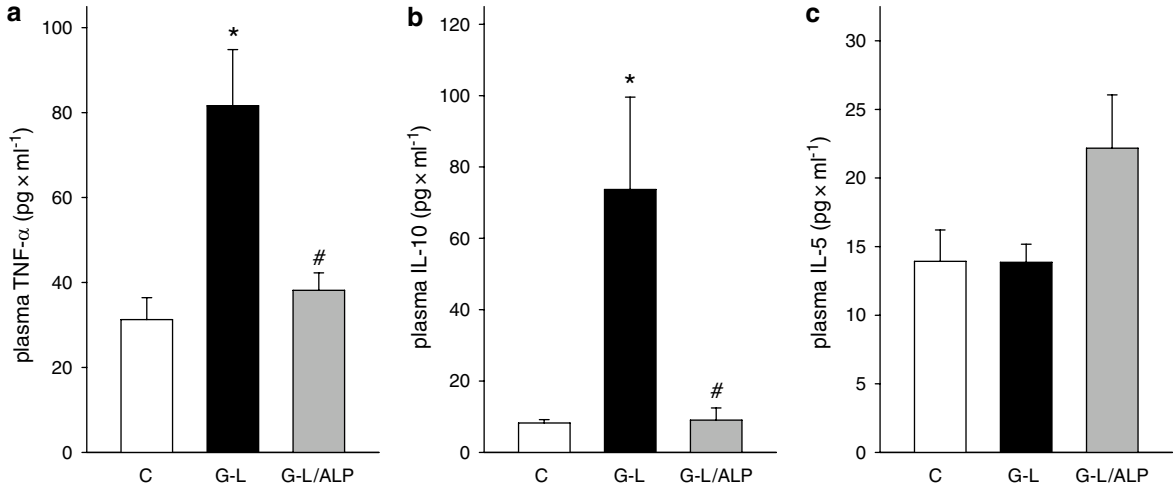

Figure 6 Plasma concentrations of TNF-α (**a**), IL-10 (**b**) and IL-5 (**c**). For induction of acute liver injury, animals were injected with D-GalN (720 mg kg^{-1} i.p.) and LPS (10 μg kg^{-1} i.p.) and received either saline (G-L) or ALP (15 mg kg^{-1}; G-L/ALP). Control animals received isotonic saline only (C). Values are given as means ± s.e.m.; ANOVA and Dunn's test; *$P<0.05$ vs C, $^{\#}P<0.05$ vs G-L.

models of endotoxemic and bacteremic shock (Oberholzer *et al*., 2002). In line with this, application of an IL-10-Fc fusion protein showed some slight tendency towards better survival in D-GalN/LPS- and ALP-treated animals. However, IL-10 does not seem to be the only protective player in this complex scenario and therefore it failed to significantly reduce lethality in our study.

It has been reported that survival and apoptotic liver injury in response to D-GalN/LPS are mainly dependent on secreted TNF-α signaling (Mignon *et al*., 1999; Nowak *et al*., 2000). Thus, one might have expected a drastic reduction of apoptosis in ALP-treated animals. However, we found a massive increase in caspase-3 activity in D-GalN/LPS-treated mice, regardless of ALP application. D-GalN, an amino sugar, is exclusively metabolized in hepatocytes, leading to a selective depletion of uridine nucleotides with severe transcription and translation arrest, as early as 30 min after injection (Leist *et al*., 1995). The depression of protein or RNA synthesis in the liver by approximately 50% sensitizes the liver towards TNF-α (Leist *et al*., 1995), which in turn activates NFκB by releasing it from the cytoplasmic inhibitor protein IκBβ (Tapalaga *et al*., 2002). Activated NFκB translocates into the nucleus with a maximal level occurring in the nuclear fraction 2.5 h after D-GalN/TNF-α administration. This is followed by an incomplete shuttle back to the cytoplasm after 4.5 h, as approximately 30% of NFκB remains in the nuclear fraction (Tapalaga *et al*., 2002). In line with this, we could observe a marked increase of NFκB-p65 immunostaining in hepatocellular nuclei at 6 h after giving D-GalN/LPS. ALP treatment significantly decreased the number of NFκB-p65 positive hepatocellular nuclei, however, without concomitant change in apoptotic cells. This might imply that apoptotic cell death is – at least in part – mediated by NFκB-independent mechanisms. Moreover, it is reasonable to assume that the cascade of apoptotic cell death was already initiated before NFκB shuttled back to the cytoplasm. Although we could demonstrate the efficacy of ALP with respect to the NFκB-dependent inflammatory process, ALP does not block apoptosis or caspase-3 levels. Since animals die from massive apoptosis and not from inflammatory tissue necrosis as indicated by lowered liver enzyme levels, it is obvious that survival is not significantly affected.

NFκB also activates cytoprotective genes of the inhibitor of apoptosis (IAP) family, which act as anti-apoptotic factors and support cell survival (Tapalaga *et al*., 2002). Nitric oxide (NO) derived from inducible NO synthase and other inhibitors of apoptosis (e.g. cIAP1 and cIAP2) are NFκB-regulated genes modulating cell survival (Dijkstra *et al*., 2002). Thus, inhibition of NFκB in ALP-treated animals may turn out to effectively decrease its anti-apoptotic potential and thus explain the failure to protect against this mode of cell injury. Besides, NFκB is essentially involved in the resolution of inflammation through regulation of leukocyte apoptosis (Lawrence *et al*., 2001). Clearance of apoptotic leukocytes by macrophages shortens the half-life of leukocytes and their harmful tissue-damaging effects. Although initial NFκB activation in leukocytes is associated with pro-inflammatory gene expression, such activation is later associated with the expression of anti-inflammatory genes and the induction of apoptosis (Lawrence *et al*., 2001). Thus, it is reasonable to hypothesize that the favorable effect of ALP, that is, local inhibition of aggressive leukocyte-dependent mediators, is neutralized by the long-lasting effect of leukocytes due to ALP-induced NFκB inhibition. Although this concept is in good agreement with our experimental data, future investigations will be needed to elucidate this interesting new issue that has emerged as a result of the present studies.

In summary, ALP effectively protected against D-GalN/LPS-associated intrahepatic inflammatory responses by inhibition of NFκB activity and offered new approaches for future developments of therapeutic agents targeting inflammatory tissue injury. However, in states of acute liver failure, where apoptotic cell death represents the predominant mode of cell death as occurs in the D-GalN/LPS-based liver injury model, a protease-inactivating approach such as delivery of ALP seems to be inadequate in improving survival.

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Conflict of interest

The authors state no conflict of interest.

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