Role of FAN in tumor necrosis factor-Δ and lipopolysaccharide-induced interleukin-6 secretion and lethality in D-galactosamine-sensitized mice

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Running title: Regulation by FAN of IL-6 secretion
Summary

TNF-induced neutral sphingomyelinase-mediated generation of ceramide, a bioactive lipid molecule, is transduced by the adaptor protein FAN which binds to the intracellular region of the CD120a TNF receptor. FAN-deficient mice do not exhibit any gross abnormality. To further explore the functions of FAN in vivo, and because CD120a-deficient mice are resistant to endotoxin-induced liver failure and lethality, we investigated the susceptibility of FAN-deficient animals to lipopolysaccharide (LPS). We show that after D-galactosamine sensitization FAN-deficient mice were partially resistant to LPS- and TNF-induced lethality. Although LPS challenge resulted in a hepatic ceramide content lower in mutant mice than in control animals, it triggered similar histological alterations, caspase activation and DNA fragmentation in the liver. Interestingly, LPS-induced elevation of IL-6 (but not TNF) serum concentrations was attenuated in FAN-deficient mice. A less pronounced secretion of IL-6 was also observed after LPS or TNF treatment of cultured peritoneal macrophages and embryonic fibroblasts isolated from FAN-deficient mice, as well as in human fibroblasts expressing a mutated FAN. Finally, we show that D-galactosamine-sensitized IL-6-deficient mice were partially resistant to endotoxin-induced liver apoptosis and lethality. These findings highlight the role of FAN and IL-6 in the inflammatory response initiated by endotoxin and implicating TNF.
Keywords: interleukin-6; TNF; ceramide; FAN; endotoxin; sphingolipid

Abbreviations: FAN, Factor Associated with Neutral sphingomyelinase activation; IL-6, Interleukin-6; LPS, lipopolysaccharide; MAPK, Mitogen-Activated Protein Kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-ΔB, Nuclear Factor-ΔB; NSD, Neutral Sphingomyelinase Domain; PMA, phorbol 12-myristate 13-acetate; TNF, Tumor Necrosis Factor-Δ.
Introduction

Tumor necrosis factor-\(\Delta\) (TNF) is a potent cytokine produced by many cell types that exerts pleiotropic functions, mediating inflammation, immunity, and modulation of cell death, differentiation and survival (1, 2). As a multifunctional proinflammatory cytokine, TNF has been implicated in the pathogenesis of a number of human disorders, including sepsis, diabetes, cancer, multiple sclerosis, and inflammatory diseases (3-5). TNF elicits its biological effects through binding to two cell surface receptors, a 60-kDa receptor (TNFRSF1A or CD120a) and a 80-kDa receptor (TNFRSF1B or CD120b), which belong to a superfamily of proteins regulating host defense, organogenesis, immune and inflammatory responses (5-8). Most of TNF activities appear to be mediated by CD120a. These are signaled through a complex network of intracellular pathways, that involve adapter proteins and lead to activation of the caspase cascade or AP-1 and NF-\(\Delta\)B transcription factors (1, 6, 7, 9). The crucial role of the TNF/CD120a system in host defense and immunity is well illustrated by the observation that mice lacking CD120a are highly susceptible to infections by intracellular pathogens, but are protected from acute hepatotoxicity induced by lipopolysaccharide (LPS) after sensitization with the liver-specific transcriptional inhibitor D-galactosamine (10, 11).

In the last decade, sphingolipids have emerged as a novel class of biomodulators which can regulate a wide spectrum of cellular responses (12-14). In particular, sphingomyelin metabolites, i.e. ceramide, sphingosine and sphingosine 1-phosphate, have been shown to signal some TNF biological effects (for reviews, see (15, 16)). Engagement of TNF with CD120a has been reported to activate both acidic and neutral sphingomyelinases, resulting in sphingomyelin degradation and formation of ceramide (17-20). This lipid
molecule and/or its metabolites are believed to mediate various TNF-induced responses, including differentiation of leukemic cells, IL-6 secretion by osteoblasts, and apoptosis of various cell types (16, 19, 21, 22). In endothelial cells, however, TNF stimulation of sphingosine kinase is accompanied by cell survival (23).

TNF-triggered activation of neutral sphingomyelinase is mediated by the adapter protein FAN (Factor Associated with Neutral sphingomyelinase activation) which binds a short motif of the intracellular part of CD120a termed NSD (Neutral Sphingomyelinase Domain) (24, 25). FAN is a member of the WD-repeat family that is required for neutral sphingomyelinase-mediated generation of ceramide following cell stimulation with TNF (25) as well as CD40 ligand (26) and cannabinoids (27). Cell culture models have demonstrated that FAN regulates TNF-induced apoptosis (28), yet does not affect other TNF effects such as activation of mitogen-activated protein kinases (MAPK) or expression of adhesion molecules (28-30). Mice harboring a targeted disruption of the gene encoding FAN exhibit no gross phenotypic abnormalities but display a delayed (possibly TNF-mediated) cutaneous barrier repair (29).

To further investigate the physiological functions of FAN, and based on the role of CD120a in the inflammatory response and liver failure, we have analyzed the response of FAN-deficient mice to LPS or TNF challenges. Here, we show that after sensitization with D-galactosamine, these knockout animals are partially resistant to LPS and TNF-induced lethality.
Experimental Procedures

Cytokines and antibodies
Recombinant human and murine TNFs were purchased from PeproTech (Tebu-Bio, Le Perray-en-Yvelines, France). Anti-CD95 (clone Jo2) was from Pharmingen (BD Biosciences, Le-Pont-de-Claix, France), and anti-α-actin from Sigma (L’Isle d’Abeau, France).

Animals and treatments
FAN-deficient (FAN –/-) mice were obtained from FAN +/- embryos (kindly provided by Drs. S. Adam and M. Krönke, University of Kiel, Germany) (29) that were implanted in C57BL/6 females (in CDTA, CNRS, Orléans, France). Embryo freezing was done exactly after 10 backcrosses into the C57BL/6 strain. Mice were genotyped by PCR using tail DNA and the following primers: Typ4 (sense), 5’-CCT TAG TTA TCT CGG TGC CAG G-3’, Typ4rev (antisense), 5’-GGA AGC CAC ACT GCT ACA CAG G-3’, and Neo1500, 5’-TCG CCT TCT ATC GCC TCC TTG-3’. Each DNA sample was amplified with primers Typ4 and Typ4rev (giving a 1 000 bp product in wild-type and heterozygous mice), and, independently, with all three primers (giving either a 1 000 bp product in wild-type or a 800 bp fragment in FAN –/- mice). FAN +/- mice obtained from heterozygous breeding pairs served as controls. IL-6-deficient mice, maintained in C57BL/6 background (31), were obtained from Drs. M. Thomsen and F. Bayard (Institut Louis Bugnard, Toulouse, France). All animals were housed under SPF conditions and maintained on a 12:12 h light-dark cycle with lights on at 07:00. Animal studies were ethically approved and carried out in agreement with national and international policies.
At the age of 7-10 weeks, mice (weighing approximately 20-25 g) were injected i.p. a single dose of D-galactosamine (20 mg; Sigma) followed by either i.p. injection of LPS (0.1-10 μg; *Salmonella minnesota*; Sigma) or i.v. injection of murine recombinant TNF (1-10 μg/kg of body weight), in a total volume of 0.1 ml of PBS or PBS containing 1% BSA, respectively. Alternatively, mice received i.p. only LPS (100 μg) or anti-CD95 antibody (0.36 μg/g of body weight) in PBS. Animals were continuously monitored for survival. Any animal that appeared moribund was euthanised to avoid undue pain and suffering. Blood was collected by retro-orbital puncture for the determination of cytokine concentrations and liver enzyme activities. Mice were sacrificed at designated time points for histology and biochemical studies under anesthesia. The right lateral lobe of the liver was preserved for routine histology, and the remainder tissue was immediately frozen in liquid nitrogen.

Histological analyses
Liver specimens were rapidly washed with saline and immediately fixed in PBS containing 4% formaldehyde. Five-μm sections were obtained from paraffin-embedded blocks, and stained with hematoxylin-eosin. Liver injury was assessed (by a blinded pathologist) by evaluating lobular necrosis, presence of apoptotic bodies, hemorrhage (peliosis hepatis), and portal inflammation.

Detection of DNA fragmentation
Frozen liver specimens (approximately 0.2 g) were homogenized with 1 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 10 mM EDTA, 0.2% Sodium Dodecyl Sulfate, and 0.5 mg of proteinase K per ml. After incubation for 1 h at 50°C, the lysates were centrifuged for 15,000 x g for 5 min. DNA in the supernatants was extracted using phenol/chloroform and
treated for 10 min at 37°C with 10 μg of RNase A to eliminate overlapping bands of RNA. Sample equivalents to 25 μg DNA were subjected to 2 % agarose gel electrophoresis, stained with ethidium bromide, and visualized by UV illumination.

Determination of caspase activity
Effector caspase activity was measured on liver lysates using the fluorogenic substrate Ac-Asp-Glu-Val-Asp-aminomethylcoumarin (DEVD-AMC; Bachem, Voisins-Le-Bretonneux, France) as described (32). Lysates were prepared by homogenizing liver specimens (approximately 100 mg) in 1 ml of ice-cold DEVDase buffer (10 mM Hepes-KOH pH 7.4, 42 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol, 0.5 % CHAPS, 1 mM PMSF and 2 μg/ml leupeptin) with a Dounce homogenizer. DEVDase activity was determined by incubating at room temperature for 30 min the 300 g supernatant with 40 μM of the substrate. Fluorescence intensity was recorded on a Jobin-Yvon spectrofluorometer at 351 and 430 nm for the excitation and emission wavelengths, respectively. Alternatively, caspase-3 cleavage was examined by Western blot using a rabbit anti-caspase-3 antibody that recognizes the cleaved forms (Cell Signaling-Ozyme, St Quentin-en-Yvelines, France).

Lipid concentration measurements
Lipids were extracted with chloroform/methanol from the liver lysates prepared for DEVDase assay. Aliquots of lipid extracts were used for determining total phospholipid and sphingomyelin contents by measuring inorganic phosphorus before and after mild alkaline methanolysis (33). Ceramide content was determined using E. coli diacylglycerol kinase (kindly provided by Drs. D. Perry and Y.A. Hannun, Charleston, SC) and [³²P]Δ-ATP (6 000 Ci/mmol; Perkin-Elmer, Villebon-sur-Yvette, France) as previously reported (34).
Cell culture

SV40-transformed fibroblasts from wild-type and FAN -/- mice were kindly provided by Dr. S. Adam (Kiel, Germany) and grown in DMEM containing Glutamax and 10% heat-inactivated fetal calf serum (Invitrogen, Cergy-Pontoise, France). Human SV40-transformed skin fibroblasts transfected with an empty vector (pcDNA3) or with a plasmid carrying a truncated version (encoding aa 703-917) of the FAN cDNA (pcDNA3-ΔFAN) were obtained as described (28). Transfected cells were maintained in the presence of G418 (0.2 mg/ml). Mouse peritoneal macrophages were collected by lavage of the peritoneal cavity with serum-free medium, and then cultured in Macrophage-SFM medium (Invitrogen). Cell viability was assessed using the MTT test. Mycoplasma contamination was checked by PCR using appropriate primers.

IL-6 and TNF assay

Cultured cells were stimulated under serum-free and non-toxic conditions. Unless otherwise stated, culture medium was collected after a 24 h incubation. Cells were harvested to determine viability and protein content (to ensure that wells contained similar cell numbers). IL-6 in serum or extracellular medium was quantified by enzyme immunoassay using a kit (Immunotech, Marseille, France) or a set (Pharmingen) for the human and murine cytokine, respectively. Murine TNF was similarly quantified using an EIA set from Pharmingen.

Other determinations
Serum alanine aminotransferase (ALAT) and aspartic aminotransferase (ASAT) activities were determined using an Ektachem 950 automatic analyzer. Cell surface expression of CD120a was assessed by flow cytometry using a rat anti-mouse CD120a antibody conjugated to R. phycoerythrin (Serotec, Cergy, France) on a FACSCalibur (Becton-Dickinson) cytometer. Protein content was determined using bicinchoninic acid.

Statistical studies

Results are expressed as means ± SD and are averages of at least three values per experiment. Mean values were compared using the Student’s t test. For animal studies, Kaplan-Meier survival rates were compared using the logrank test. When 75 % or 50 % survival times were compared, the Student’s t test was employed.
Results

D-Galactosamine-sensitized FAN-deficient mice partially resist LPS

Sensitivity of mice to LPS injection after sensitization with D-galactosamine is known to critically depend on functional TNF/CD120a signaling (10, 11). Because FAN has been reported to transduce some TNF signals through CD120a, we tested the susceptibility of FAN-deficient mice to titrated doses of LPS. FAN-deficient and wild-type mice were injected i.p. with varying doses of LPS, and survival was monitored. As previously observed, mice were unsensitive to LPS (100 μg) in the absence of pretreatment with D-galactosamine (Table 1). As indicated in Table 1 and illustrated in Fig. 1, after sensitization with D-galactosamine FAN-deficient animals were more resistant to the lethal effects of low doses (0.1-10 μg) of LPS than their control counterparts.

Because liver injury is viewed as the primary factor responsible for LPS-induced lethality, and because hepatic damage is due to apoptosis of hepatocytes (35, 36), we analyzed histologically and biochemically the livers of FAN-deficient and wild-type mice challenged with D-galactosamine and LPS. No significant genotype-dependent differences could be morphologically observed in the extent of parenchymal damage characterized by apoptosis, caspase activation (as assessed by DEVDase activity or, alternatively, immunoblotting for caspase-3), and DNA fragmentation (Fig. 2). Accordingly, serum ALAT and ASAT activities were similarly elevated in the two animal groups (data not shown).

However, the ceramide content of the livers from FAN-deficient mice was lower than that found in the livers from control animals (Fig. 2E), although still being elevated as compared to untreated mice (the ceramide to sphingomyelin ratio reached 166 % and 257 % in FAN-deficient and wild-type mice, respectively). This finding is consistent with the notion that
FAN is required for CD120a-mediated formation of ceramide via activation of neutral sphingomyelinase (29).

D-Galactosamine-sensitized FAN-deficient mice are also partially resistant to TNF

Because TNF is considered as a central mediator of the LPS-induced liver injury and lethal toxicity (37-41), we next studied the susceptibility of FAN-deficient mice to the toxic effects of murine TNF after pretreatment with D-galactosamine. As shown in Fig. 3, FAN knockout mice were less sensitive to rather low doses (2.5 to 5 μg/kg) of TNF than wild-type mice. This partial resistance was overcome by increasing the TNF dose (Fig. 3). Moreover, a comparable susceptibility of mutant and control mice to the lethal effects of an anti-CD95 agonist antibody was seen (data not shown), indicating that not all death receptor pathways are affected in FAN-deficient animals. As observed for LPS-induced liver injury, hepatic damage (as assessed by histopathology and measurement of serum ALAT and ASAT activities), caspase activation and DNA fragmentation were essentially the same in FAN-deficient and wild-type mice (data not shown).

Impaired IL-6 secretion in FAN-deficient mice

The inflammatory response initiated by LPS and mediated by TNF implicates the production and release of other cytokines such as IL-6 (6, 42). As a potential mediator of TNF, ceramide has been shown to stimulate IL-6 secretion (e.g. (22, 43)). Because FAN regulates TNF-induced ceramide generation, we investigated whether IL-6 secretion can be modulated by FAN. First, we measured IL-6 in the serum of FAN-deficient and wild-type mice that were challenged with LPS. Injection of 10 μg LPS in D-galactosamine-sensitized wild-type mice resulted in a robust IL-6 secretion that peaked at about 2.5 h (Fig. 4B).
Serum IL-6 levels were markedly lower (up to 2.6 fold) in FAN-deficient mice. When mice were challenged with 100 μg LPS alone, serum IL-6 levels were also reduced in the mutant mice (Fig. 5B). Of note, serum TNF levels were similar in the two animal groups (Figs. 4A and 5A). In the serum from animals injected with TNF, IL-6 levels in mutant mice were 36 ± 5 % lower than in wild-type animals (n=18 mice), where the levels averaged 480 and 790 pg/ml at 2 and 3.5 hours, respectively.

Secondly, IL-6 secretion by cultured murine cells was assessed. Peritoneal macrophages were isolated from FAN-deficient and wild-type mice and treated with LPS. As shown in Fig. 6A, mutant macrophages secreted less IL-6 than their normal counterparts. IL-6 secretion by FAN-deficient resident peritoneal macrophages was also lower than that of control cells after challenge with 50 ng/ml TNF (IL-6 secretion was 49 ± 32 and 11 ± 20 pg/ml in wild-type and mutant cells (n=10 mice; p<0.05). A similar difference was noted on thioglycolate-elicited peritoneal macrophages (data not shown). We then tested murine embryonic fibroblasts treated with TNF. Again, mutant cells secreted less IL-6 than their normal counterparts (Fig. 6B). Interestingly, human and murine TNF gave comparable results, indicating that these effects are mediated by CD120a because human TNF specifically binds murine CD120a (6). Of note, mutant and wild-type murine macrophages and fibroblasts expressed at their surface similar levels of CD120a as demonstrated by flow cytometry (data not shown). Finally, IL-6 secretion by human skin fibroblasts overexpressing a truncated, dominant-negative form of FAN was found to be lower than that in control fibroblasts (Fig. 6C). However, FAN-deficient and control cells responded similarly to PMA (Fig. 6). Collectively, these data suggest that FAN-deficient cells, which generate less ceramide in response to TNF (28, 29), have an impaired ability to secrete IL-6 when challenged with TNF or LPS.
D-Galactosamine-sensitized IL-6-deficient mice are partially resistant to LPS

IL-6 is a multifaceted cytokine with both pro- and anti-inflammatory properties (44). To test whether a defective IL-6 secretion could account for a partial protection against endotoxin under D-galactosamine sensitization conditions, IL-6-deficient mice which are homozygous for a disruption in the second exon of IL-6 gene (31), were challenged with LPS. As shown in Fig. 7A, these mutant animals were partially protected from the lethal effects of LPS. Whereas serum IL-6 remained undetectable in these mice (Fig. 7C), serum TNF levels were comparable to those of control mice (Fig. 7B).

Histologically, liver apoptosis was less prominent in IL-6-deficient mice (Fig. 8A). In addition, DEVDase activation was less pronounced (Fig. 8B) and DNA degradation strongly attenuated (Fig. 8C) in mice lacking IL-6. Accordingly, serum transaminase activities were lower in mutant animals (data not shown). However, of special interest was the finding that hepatic ceramide content was comparably elevated in control and mutant mice (Fig. 8D; see Fig. 2E for comparison). These observations suggest that, in D-galactosamine-sensitized mice, IL-6 plays a protective role against the (hepato)toxicity of endotoxin without affecting ceramide production.
Discussion

Acute liver failure (or fulminant hepatitis) can occur in several pathological conditions, including viral hepatitis, sepsis, ischemia, metabolic disorders, and poisoning by various toxins or drugs (45). In mice, these situations have often been mimicked by the administration of endotoxin or TNF following sensitization with D-galactosamine. TNF is indeed known to play a pivotal role in liver homeostasis and many of these hepatotoxic processes (37-41, 46), although their underlying signaling pathways remain poorly elucidated. Understanding the signal transduction mechanisms of hepatocellular damage and the lethal effects of LPS or TNF is therefore of crucial importance for developing potential appropriate therapies of liver diseases.

This study addressed the specific contribution of FAN, an adapter protein regulating some cytotoxic effects of TNF, to the pathogenesis of LPS or TNF-induced lethality. Evidence is provided that mice with a genetic deletion of FAN are partially resistant to these challenges. In addition, mice lacking FAN (or cells derived thereof) produced less ceramide and IL-6 than their control littermates when challenged with endotoxin, suggesting a connection between IL-6 secretion and responsiveness to LPS. As a matter of fact, IL-6-deficient animals were partially resistant to LPS-induced liver damage and lethality.

Numerous reports have documented the production of the bioactive sphingolipid ceramide in response to TNF, both in cell culture models and in vivo (for a recent review, see (16)). With regard to the potential role of ceramide in liver apoptosis, TNF has been described to trigger ceramide generation in cultured hepatocytes as well as in the liver of D-galactosamine-sensitized mice (47, 48), and exogenous ceramide elicits hepatocyte
apoptosis (49). Hepatic ceramide content is also increased after LPS injection (48, 50), a condition previously reported to result in ceramide accumulation in several other tissues (51, 52). Moreover, ceramide levels are elevated in the hepatocyte nuclei following portal vein branch ligation (53).

Accumulation of ceramide or its metabolic derivative GD3 ganglioside has been proposed to mediate the toxic effects of TNF or LPS based on the resistance of mice lacking acid sphingomyelinase (48, 51). LPS has even been suggested to mimick ceramide (54). The present study indicates that the ceramide produced by neutral sphingomyelinase, which is regulated by FAN, may also participate to the TNF or LPS toxicity. Whether the generation of this putative sphingolipid mediator in the liver is specifically and uniquely responsible for hepatocyte apoptosis and subsequent systemic demise after injection of endotoxin is not yet established. Rather, the observation that FAN-deficient mice are partially protected from the lethal effects of LPS and TNF despite induction of cell death in the liver suggests that (neutral sphingomyelinase-derived) ceramide is only part of the signaling pathways leading to apoptosis in liver. One could postulate that optimal cell death induction occurs when both acid and neutral sphingomyelinsases are being activated. The fact that ceramide was equally elevated in the liver of control and IL-6-deficient mice would suggest that ceramide is not sufficient for induction of hepatic apoptosis. Another possibility is that ceramide or its metabolites regulate other pathways, not exclusively in liver, that eventually lead to animal death or survival. Interestingly, elevated ceramide levels in mononuclear cells have been found to correlated with TNF levels and severity of sepsis (55), indicating that sphingolipids may also regulate some other aspects of the inflammatory response.

Because of the important role of TNF in inflammation, including secretion of interleukins such as IL-6 (6), and because the cytotoxic effect and IL-6 production elicited by TNF are
two closely linked responses (56), we evaluated the role of FAN in the secretion of IL-6. Experimental evidence is provided here that FAN can indeed regulate IL-6 secretion. Not only cultured cells isolated from mice lacking FAN but also cells overexpressing a dominant-negative form of FAN exhibited a defect in IL-6 secretion. This impairment was also noticed when serum cytokine concentration was measured after LPS challenge. This suggests that the ceramide produced by neutral sphingomyelinase can stimulate IL-6 secretion. Either ceramide or its metabolites has been described to enhance IL-6 gene expression and protein production in different cell types, including fibroblasts (43, 57), osteoblasts (22, 58, 59), epithelial cells (60, 61), and human astrocytoma cells (62). In some instances, the ceramide metabolite sphingosine 1-phosphate was proposed as the mediator for cytokine induction (58). The fact that FAN (and the NSD of CD120a) regulates only partially IL-6 secretion does not disagree with previous observations indicating that the death domain of CD120a is sufficient for IL-6 gene induction (63). As for induction of cytotoxicity (28), it is conceivable that the NSD and death domain cooperate to give a full response.

How sphingolipids and FAN regulate IL-6 secretion is still unclear. TNF is known to trigger IL-6 production by increased NF-ΔB-mediated gene transcription, the activation of NF-ΔB likely implicating MAPK, and in particular p38 (42, 64), as well as reactive oxygen species (65). However, TNF-induced activation of the ERK type kinases (28-30) and p38 (Malagarie-Cazenave, Ségui, and Levade, unpublished) appears independent of FAN. Whether NF-ΔB activation and subsequent IL-6 secretion is modulated by FAN through production of reactive oxygen species is currently under investigation. Interestingly, LPS
has very recently been reported to stimulate neutral sphingomyelinase activity which is mandatory for induction of inducible NO-synthase and NF-κB activation (66).

IL-6 is a multifaceted cytokine which, just as TNF (2, 67), can show a functional duality in liver, promoting either apoptosis and injury, or survival and regeneration (44, 68). The action of TNF and IL-6 on liver as two-edge swords might be related to the activation state of transcription factors (45). On the one hand, IL-6 can confer protection against some liver insults such as CD95 or concanavalin-A. Pretreatment of mice with an antibody to IL-6 prior to administration of LPS or enterotoxin and D-galactosamine enhanced mortality, while pretreatment with IL-6 reduced death (69, 70). On the other hand, IL-6 acts as a pro-inflammatory cytokine in the context of endotoxemia and acute liver failure. It is well known that IL-6 serum levels correlate with severity of sepsis (71, 72). LPS failed to induce fever response in IL-6-deficient mice (73). Furthermore, increased sensitivity to LPS in some mutant mice has been related to high circulating levels of IL-6 (74). IL-6 can also enhance TNF-induced apoptosis of hepatocytes sensitized by actinomycin D (75). However, IL-6-deficient mice appeared equally sensitive to TNF even after sensitization with D-galactosamine (76). Hence, the contribution of IL-6 to the animal models of acute liver failure represented by injection of LPS or TNF to D-galactosamine-sensitized mice is not clear. Our observations on knockout mice argue for a role of IL-6 in the development of the lethal effects of LPS. Interestingly, whereas IL-6-deficient mice displayed less apoptosis in the liver and survived longer than control animals, the hepatic content of ceramide was equally increased. This strengthens the notion that ceramide generation occurs upstream of IL-6 gene expression, and that IL-6 may exert pro-inflammatory effects that ultimately contribute to lethality induced by LPS and D-galactosamine. Whether the liver is the only IL-6 target that mediates lethality needs to be determined.
In summary, FAN appears to contribute to the pathogenesis of the fulminant hepatitis triggered by endotoxin, possibly by regulating IL-6 secretion. Whether ceramide is the only mediator of these effects, whether FAN also regulates the production of other cytokines such as IL-8 or GM-CSF secreted in response to TNF (64), whether FAN modulates other LPS-induced responses than those mediated by TNF, and by which molecular mechanisms remain to be elucidated. Clarifying these issues may help developing novel strategies for treatment of diseases characterized by acute liver dysfunction implicating TNF.
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References

1. Chen, G., and Goeddel, D. V. (2002) *Science* **296**, 1634-1635
2. Wajant, H., Pfizenmaier, K., and Scheurich, P. (2003) *Cell Death Differ* **10**, 45-65
3. Tracey, K. J., and Cerami, A. (1993) *Annu. Rev. Cell Biol.* **9**, 317-343
4. McDermott, M. F. (2001) *Cell Mol Biol (Noisy-le-grand)* **47**, 619-635
5. Aggarwal, B. B. (2003) *Nat Rev Immunol* **3**, 745-756
6. Vandenabeele, P., Declercq, W., Beyaert, R., and Fiers, W. (1995) *Trends Cell Biol.* **5**, 392-399
7. Wallach, D., Varfolomeev, E. E., Malinin, N. L., Goltsev, Y. V., Kovalenko, A. V., and Boldin, M. P. (1999) *Annu. Rev. Immunol.* **17**, 331-367
8. Locksley, R. M., Killeen, N., and Lenardo, M. J. (2001) *Cell* **104**, 487-501
9. Baud, V., and Karin, M. (2001) *Trends Cell Biol* **11**, 372-377
10. Pfeffer, K., Matsuyama, T., Kundig, T. M., Wakeham, A., Kishihara, K., Shahinian, A., Wiegmann, K., Ohashi, P. S., Kronke, M., and Mak, T. W. (1993) *Cell* **73**, 457-467
11. Rothe, J., Lesslauer, W., Lotscher, H., Lang, Y., Koebel, P., Kontgen, F., Althage, A., Zinkernagel, R., Steinmetz, M., and Bluethmann, H. (1993) *Nature* **364**, 798-802
12. Hannun, Y. (1996) *Science* **274**, 1855-1859
13. Kolesnick, R. N., and Krönke, M. (1998) *Annu. Rev. Physiol.* **60**, 643-665
14. Hannun, Y. A., and Obeid, L. M. (2002) *J Biol Chem* **277**, 25847-25850
15. Kolesnick, R., and Golde, D. W. (1994) *Cell* **77**, 325-328
16. Malagarie-Cazenave, S., Andrieu-Abadie, N., Séguin, B., Gouazé, V., Tardy, C., Cuvillier, O., and Levade, T. (2002) *Exp. Rev. Mol. Med.* **20** December, [http://www.expertreviews.org/0200546X](http://www.expertreviews.org/0200546X)
17. Kim, M., Linardic, C., Obeid, L., and Hannun, Y. (1991) *J. Biol. Chem.* **266**, 484-489

18. Dressler, K. A., Mathias, S., and Kolesnick, R. N. (1992) *Science* **255**, 1715-1718

19. Obeid, L. M., Linardic, C. M., Karolak, L. A., and Hannun, Y. A. (1993) *Science* **259**, 1769-1771

20. Wiegmann, K., Schütze, S., Machleidt, T., Witte, D., and Krönke, M. (1994) *Cell* **78**, 1005-1015

21. Adam-Klages, S., Schwandner, R., Adam, D., Kreder, D., Bernardo, K., and Krönke, M. (1998) *J. Leukoc. Biol.* **63**, 678-682

22. Shinoda, J., Kozawa, O., Tokuda, H., and Uematsu, T. (1999) *Cell Signal* **11**, 435-441

23. Xia, P., Wang, L., Gamble, J. R., and Vadas, M. A. (1999) *J Biol Chem* **274**, 34499-34505

24. Adam, D., Wiegmann, K., Adam-Klages, S., Ruff, A., and Krönke, M. (1996) *J. Biol. Chem.* **1996**, 14617-14622

25. Adam-Klages, S., Adam, D., Wiegmann, K., Struve, S., Kolanus, W., Schneider-Mergener, J., and Krönke, M. (1996) *Cell* **86**, 937-947

26. Ségui, B., Andrieu-Abadie, N., Adam-Klages, S., Meilhac, O., Kreder, D., Garcia, V., Bruno, A. P., Jaffrézou, J. P., Salvayre, R., Krönke, M., and Levade, T. (1999) *J. Biol. Chem.* **274**, 37251-37258

27. Sanchez, C., Rueda, D., Segui, B., Galve-Roperh, I., Levade, T., and Guzman, M. (2001) *Mol Pharmacol* **59**, 955-959
28. Ségui, B., Cuvillier, O., Adam-Klages, S., Garcia, V., Malagarie-Cazenave, S., Lévêque, S., Caspar-Bauguil, S., Coudert, J., Salvayre, R., Krönke, M., and Levade, T. (2001) *J. Clin. Invest.* **108**, 143-151

29. Kreder, D., Krut, O., Adam-Klages, S., Wiegmann, K., Scherer, G., Plitz, T., Jensen, J. M., Proksch, E., Steinmann, J., Pfeffer, K., and Krönke, M. (1999) *EMBO J.* **18**, 2472-2479

30. Lüschen, S., Adam, D., Ussat, S., Kreder, D., Schneider-Brachert, W., Krönke, M., and Adam-Klages, S. (2000) *Biochem. Biophys. Res. Commun.* **274**, 506-512

31. Kopf, M., Baumann, H., Freer, G., Freudenberg, M., Lamers, M., Kishimoto, T., Zinkernagel, R., Bluethmann, H., and Kohler, G. (1994) *Nature* **368**, 339-342

32. Cuvillier, O., Edsall, L., and Spiegel, S. (2000) *J. Biol. Chem* **275**, 15691-15700

33. Andrieu, N., Salvayre, R., and Levade, T. (1994) *Biochem. J.* **303**, 341-345

34. Bielawska, A., Perry, D. K., and Hannun, Y. A. (2001) *Anal Biochem* **298**, 141-150

35. Leist, M., Gantner, F., Bohlinger, I., Tiegs, G., Germann, P. G., and Wendel, A. (1995) *Am J Pathol* **146**, 1220-1234

36. Jaeschke, H., Fisher, M. A., Lawson, J. A., Simmons, C. A., Farhood, A., and Jones, D. A. (1998) *J Immunol* **160**, 3480-3486

37. Lehmann, V., Freudenberg, M. A., and Galanos, C. (1987) *J Exp Med* **165**, 657-663

38. Tiegs, G., Wolter, M., and Wendel, A. (1989) *Biochem Pharmacol* **38**, 627-631

39. Morikawa, A., Sugiyama, T., Kato, Y., Koide, N., Jiang, G. Z., Takahashi, K., Tamada, Y., and Yokochi, T. (1996) *Infect Immun* **64**, 734-738

40. Bradham, C. A., Plumpe, J., Manns, M. P., Brenner, D. A., and Trautwein, C. (1998) *Am J Physiol* **275**, G387-392
41. Nowak, M., Gaines, G. C., Rosenberg, J., Minter, R., Bahjat, F. R., Rectenwald, J., MacKay, S. L., Edwards, C. K., 3rd, and Moldawer, L. L. (2000) *Am J Physiol Regul Integr Comp Physiol* **278**, R1202-1209

42. Vanden Berghe, W., Vermeulen, L., De Wilde, G., De Bosscher, K., Boone, E., and Haegeman, G. (2000) *Biochem Pharmacol* **60**, 1185-1195

43. Launderkind, S. J., Bielawska, A., Raghow, R., Hannun, Y. A., and Ballou, L. R. (1995) *J Exp Med* **182**, 599-604

44. Naka, T., Nishimoto, N., and Kishimoto, T. (2002) *Arthritis Res* **4 Suppl 3**, S233-242

45. Galun, E., and Axelrod, J. H. (2002) *Biochim Biophys Acta* **1592**, 345-358

46. Streetz, K., Leifeld, L., Grundmann, D., Ramakers, J., Eckert, K., Spengler, U., Brenner, D., Manns, M., and Trautwein, C. (2000) *Gastroenterology* **119**, 446-460

47. Osawa, Y., Banno, Y., Nagaki, M., Nozawa, Y., Moriwaki, H., and Nakashima, S. (2001) *Liver* **21**, 309-319

48. Garcia-Ruiz, C., Colell, A., Mari, M., Morales, A., Calvo, M., Enrich, C., and Fernandez-Checa, J. C. (2003) *J Clin Invest* **111**, 197-208.

49. Jones, B. E., Lo, C. R., Srinivasan, A., Valentino, K. L., and Czaja, M. J. (1999) *Hepatology* **30**, 215-222

50. Deaciuc, I. V., Nikolova-Karakashian, M., Fortunato, F., Lee, E. Y., Hill, D. B., and McClain, C. J. (2000) *Alcohol Clin Exp Res* **24**, 1557-1565

51. Haimovitz-Friedman, A., Cordon-Cardo, C., Bayoumy, S., Garzotto, M., McLoughlin, M., Gallily, R., Edwards, C. K., Schuchman, E. H., Fuks, Z., and Kolesnick, R. (1997) *J Exp Med* **186**, 1831-1841
52. Memon, R. A., Holleran, W. M., Moser, A. H., Seki, T., Uchida, Y., Fuller, J., Shigenaga, J. K., Grunfeld, C., and Feingold, K. R. (1998) Arterioscler Thromb Vasc Biol 18, 1257-1265

53. Tsugane, K., Tamiya-Koizumi, K., Nagino, M., Nimura, Y., and Yoshida, S. (1999) J Hepatol 31, 8-17

54. Wright, S. D., and Kolesnick, R. N. (1995) Immunol Today 16, 297-302

55. Delogu, G., Famularo, G., Amati, F., Signore, L., Antonucci, A., Trinchieri, V., Di Marzio, L., and Cifone, M. G. (1999) Crit Care Med 27, 2413-2417

56. Vandevoorde, V., Haegeman, G., and Fiers, W. (1992) FEBS Lett 302, 235-238

57. Tominaga, K., Kirikae, T., and Nakano, M. (1997) Mol Immunol 34, 1147-1156

58. Kozawa, O., Suzuki, A., Kaida, T., Tokuda, H., and Uematsu, T. (1997) J Biol Chem 272, 25099-25104

59. Tokuda, H., Kozawa, O., Harada, A., and Uematsu, T. (1999) J Cell Biochem 72, 262-268

60. Hedlund, M., Svensson, M., Nilsson, A., Duan, R. D., and Svanborg, C. (1996) J Exp Med 183, 1037-1044

61. Hedlund, M., Duan, R. D., Nilsson, A., and Svanborg, C. (1998) Mol Microbiol 29, 1297-1306

62. Fiebich, B. L., Lieb, K., Berger, M., and Bauer, J. (1995) J Neuroimmunol 63, 207-211

63. Vandevoorde, V., Haegeman, G., and Fiers, W. (1997) J. Cell Biol. 137, 1627-1638

64. Beyaert, R., Cuenda, A., Vanden Berghe, W., Plaisance, S., Lee, J. C., Haegeman, G., Cohen, P., and Fiers, W. (1996) EMBO J 15, 1914-1923
65. Schulze-Osthoff, K., Beyaert, R., Vandevoorde, V., Haegeman, G., and Fiers, W. (1993) *EMBO J* **12**, 3095-3104

66. Amtmann, E., Baader, W., and Zoller, M. (2003) *Drugs Exp Clin Res* **29**, 5-13

67. Plumpe, J., Streetz, K., Manns, M. P., and Trautwein, C. (1999) *Ital J Gastroenterol Hepatol* **31**, 235-243

68. Taub, R. (2003) *J Clin Invest* **112**, 978-980

69. Barton, B. E., and Jackson, J. V. (1993) *Infect Immun* **61**, 1496-1499

70. Barton, B. E., Shortall, J., and Jackson, J. V. (1996) *Infect Immun* **64**, 714-718

71. Damas, P., Ledoux, D., Nys, M., Vrindts, Y., De Groote, D., Franchimont, P., and Lamy, M. (1992) *Ann Surg* **215**, 356-362

72. Casey, L. C., Balk, R. A., and Bone, R. C. (1993) *Ann Intern Med* **119**, 771-778

73. Chai, Z., Gatti, S., Toniatti, C., Poli, V., and Bartfai, T. (1996) *J Exp Med* **183**, 311-316

74. Nakamura, A., Mori, Y., Hagiwara, K., Suzuki, T., Sakakibara, T., Kikuchi, T., Igarashi, T., Ebina, M., Abe, T., Miyazaki, J., Takai, T., and Nukiwa, T. (2003) *J Exp Med* **197**, 669-674

75. Boer, U., Fennekohl, A., and Puschel, G. P. (2003) *J Hepatol* **38**, 728-735

76. Libert, C., Takahashi, N., Cauwels, A., Brouckaert, P., Bluethmann, H., and Fiers, W. (1994) *Eur J Immunol* **24**, 2237-2242
Table 1. Susceptibility of FAN -/- mice to the lethal effects of LPS.

| LPS (μg/mice) | GalN (mg/mice) | FAN +/+ (dead vs. injected animals) | FAN -/- (dead vs. injected animals) | p     |
|--------------|---------------|------------------------------------|-------------------------------------|-------|
| 0            | 20            | ND                                 | 0/2                                 | ND    |
| 0.1          | 20            | 5/10                               | 2/7                                 | < 0.01|
| 1            | 20            | 7/9                                | 4/7                                 | n.s.  |
| 10           | 20            | 14/18                              | 4/15                                | < 0.01|
| 10           | 0             | ND                                 | 0/2                                 | ND    |
| 100          | 0             | 0/6                                | 0/6                                 | n.s.  |

Mice received i.p. D-galactosamine (GalN) and/or LPS, and lethality was monitored up to 72 hours. ND, not determined. The differences in 75 % survival times of the two animal groups were statistically studied by actuarial analysis (n.s., not significant).
Legends to the Figures

Figure 1. Mice survival after i.p. injection of LPS (10 μg/mice) and D-galactosamine (20 mg). The number of mice is given in the plot. The logrank test indicated a statistically significant difference (p < 0.01) in survival between the two animal groups.

Figure 2. Induction of apoptosis in livers of D-galactosamine-sensitized wild-type and FAN-deficient mice treated with LPS. Liver specimens were harvested 5-6 h after LPS (10 μg) injection. A, Histological analysis of hematoxylin-eosin stained liver sections taken from wild-type (a and c) and FAN-deficient (b and d) mice treated with (c and d) or without (a and b) D-galactosamine (GalN) and LPS. Shown are representative sections from at least 11 mice of each genotype (magnification x 400). B, DEVDase activity in liver specimens. C, Western blot of cleaved forms of caspase-3. D, Analysis of DNA fragmentation. Liver genomic DNA was subjected to agarose gel electrophoresis and stained with ethidium bromide (M, DNA marker). Samples from untreated animals showed no laddering, but sometimes smeary, pattern (data not shown). E, Hepatic content of ceramide in FAN +/+ and FAN -/- mice. Liver concentration of ceramide was determined using the diacylglycerol kinase assay, and is expressed as the ratio to liver sphingomyelin concentration. In control mice, concentrations of sphingomyelin and ceramide averaged 4052 and 190 nmol/g of fresh tissue, respectively (i.e. 104 and 4.9 nmol/μmol of lipid phosphorus). The number of mice and the p value for statistical analysis are indicated in the plots.

Figure 3. Mice survival after i.p. injection of D-galactosamine (20 mg) followed by i.v. injection of murine TNF (1-10 μg/kg of body weight). Numbers of mice are given in the
plots. The logrank test indicated a statistically significant difference (p < 0.03) in survival between the two animal groups for the 3.5 μg/kg TNF dose, but not for the other doses. However, actuarial analysis of the 75 % (as well as 50 %) survival times showed a significant difference (p < 0.01) between the two animal groups for the 2.5, 3.5, and 5 μg/kg TNF doses.

Figure 4. Serum concentrations of TNF (A) and IL-6 (B) in FAN +/+ and FAN -/- mice after challenge with D-galactosamine (20 mg) and LPS (10 μg). Cytokine concentrations were determined at the indicated times post-injection. Each value corresponds to the mean ± SD on 8 to 23 mice. The p values for statistical analyses are indicated in the plot.

Figure 5. Serum concentrations of TNF (A) and IL-6 (B) in FAN +/+ and FAN -/- mice after challenge with LPS (100 μg) alone. Cytokine concentrations were determined at the indicated times post-injection. Each value corresponds to the mean ± SD on at least 4 mice. The p values for statistical analyses are indicated in the plot.

Figure 6. TNF- and LPS-induced IL-6 production by murine peritoneal macrophages (A), murine embryonic fibroblasts (B), and human skin fibroblasts (C) transfected with an empty vector (pcDNA3) or a construct encoding a truncated FAN (ΔFAN). Cells were grown in serum-free medium and stimulated for 24 hours in the absence (Con) or presence of LPS (10 μg/ml), TNF (50 ng/ml and 10 ng/ml for the human and murine cytokine, respectively), or PMA (200 nM). IL-6 concentration in the culture medium was measured by immunoassay. Determinations were performed in triplicate on the culture medium of cells.
from at least three different mice or three independent experiments. The p values for statistical analyses are indicated in the plots.

Figure 7. Susceptibility of D-galactosamine-sensitized IL-6-deficient mice to LPS. FAN +/+ animals were used as controls. A, Survival of mice after i.p. injection of D-galactosamine (20 mg) and LPS (10 μg). The number of mice is given in the plot. The logrank test indicated a statistically significant difference (p < 0.01) in survival between the two animal groups. Serum concentrations of TNF (B) and IL-6 (C) in control and IL-6-deficient mice 1 h after challenge with D-galactosamine and LPS. The number of mice and p values for statistical analyses are indicated in the plots.

Figure 8. Induction of apoptosis in livers of D-galactosamine-sensitized IL-6-deficient and control mice treated with LPS. Liver specimens were harvested 5-6 h after LPS (10 μg) injection. A, Histological analysis of hematoxylin-eosin stained liver sections taken from control (a and c) and IL-6-deficient (b and d) mice treated with (c and d) or without (a and b) D-galactosamine (GalN) and LPS. Shown are representative sections from at least 11 mice of each genotype (magnification x 250). B, DEVDase activity in liver specimens. C, Analysis of liver DNA fragmentation. D, Hepatic content of ceramide, as expressed as the ratio to liver sphingomyelin concentration. The number of mice and p values for statistical analyses are indicated in the plots.
Figure 1

Survival (%) vs. Time (h)

- FAN +/+ (n=18)
- FAN -/- (n=15)
A

FAN +/+ FAN -/-

Untreated  

+ LPS  + GalN

B

DEVDase activity (nmol/h.mg)

C

Casp-3

β-actin

D

FAN +/+ FAN -/-

M 1 2 3 4 5 6 7 8 9 10 11 12

E

Ceramide/sphingomyelin ratio

n=11 n=12

<0.001

Figure 2
Figure 3
Figure 4

A

Serum TNF (ng/ml)

- O FAN +/+  
- ■ FAN +/-

Time (h)

B

Serum IL-6 (ng/ml)

<0.005

<0.05

Time (h)
Figure 5

A

Serum TNF (ng/ml)

0 1 2 3 4 5 6

Time (h)

FAN +/+  
FAN -/-

B

Serum IL-6 (ng/ml)

0 1 2 3 4 5 6

Time (h)

=0.05
Figure 6

A

secreted IL-6 (ng/ml)

Con hTNF mTNF PMA

FAN +/+
FAN -/-

<0.05

B

secreted IL-6 (pg/ml)

Con hTNF mTNF PMA

FAN +/+ FAN -/-

<0.001

C

secreted IL-6 (ng/ml)

Con hTNF PMA

pcDNA3 ΔFAN

<0.01
Figure 7
Figure 8

A

Wild-type  IL-6 -/-

Untreated

+ LPS  + GalN

B

<0.01

DEV/Dase activity (nmol/h.mg)

C

Wild-type  IL-6 -/-

M  1  2  3  4  5  1  2  3  4  5

D

Ceramide/sphingomyelin ratio

Figure 8
Role of FAN in tumor necrosis factor-alpha and lipopolysaccharide-induced interleukin-6 secretion and lethality in D-galactosamine-sensitized mice
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