

Mice lacking TNF α receptors 1 and 2 are resistant to death and fulminant liver injury induced by agonistic anti-Fas antibody

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Abstract

The liver is particularly susceptible to Fas-mediated cytotoxicity. Mice given an adequate parenteral dose of agonistic anti-Fas antibody (aFas) or of FasL are known to develop a devastating liver injury and to die in a few hours. The present work shows that mice lacking TNFR1 and TNFR2 (R^-) both survive a single dose of aFas, otherwise rapidly lethal, and develop a mild form of hepatic damage, compared to the much more severe liver injury that in a few hours strikes wild-type mice (R^+), eventually involving increased activity of proteases of different families (caspase 3-, 8-, and 9-like, calpains, cathepsin B). Neither the overall tissue levels of Fas and FasL nor Fas expression at the hepatocyte surface are altered in the liver of R^- animals. The DNA-binding activity of the NF- κ B transcription factor is enhanced after aFas treatment, but much more markedly in R^- than in R^+ mice. Bcl2, while unchanged in untreated animals, is markedly upregulated in R^- but not in R^+ mice challenged with aFas. The requirement of a normal TNFR1/TNFR2 phenotype for full deployment of the general and liver-specific aFas toxicity in mice most likely implies that treatment with aFas in some way results in activation of the TNF α -TNFRs system and that this activation synergizes with Fas-mediated signals in causing the fulminant liver injury and the animal death. The precise cellular and molecular details underlying this interplay between Fas- and TNFRs-mediated signaling systems in the general and liver-specific aFas toxicity largely remain to be clarified.

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Abbreviations: aFas, agonistic anti-Fas antibody; R^+ , normal mice; R^- , mice knocked out for TNF-R1 and TNF-R2

Introduction

Fas, also known as CD95, Apo1, or TNFRSF6, is a type I transmembrane protein belonging to the tumor necrosis factor receptor superfamily (TNFRSF). Although also reported to convey survival/proliferation signals,^{1–3} Fas is canonically viewed as a death receptor (reviewed by Budd³, Nagata and Gostein⁴). Fas engagement and oligomerization by its ligand (FasL, TNFSF6) or by agonistic antibodies result in recruitment of the adapter protein FADD and procaspase-8 onto the death domain (DD) in its cytoplasmic tail. Proteolytic autoactivation of caspase-8 is followed by processing of effector procaspases into their active forms, leading to apoptosis execution. In some cells, categorized as type I, this 'extrinsic' pathway is adequate to perform Fas-induced apoptosis, while type II cells require activation of the 'intrinsic' or mitochondrial pathway, mainly via a truncated form of Bid (tBid) generated by caspase-8.^{5,6}

In the liver, Fas-mediated apoptosis is thought to be involved in hepatocyte homeostasis and to play important roles in a variety of diseases (reviewed by Pinkoski *et al.*⁷). Fas expression on the hepatocyte surface is increased in acute liver failure⁸ or in chronic viral hepatitis B or C, positively correlating with the severity of the process.^{9,10} In addition, high circulating levels of FasL and soluble Fas have been detected in viral or autoimmune chronic hepatitis, cirrhosis, or toxic hepatitis.^{4,11–13} The liver indeed is particularly susceptible to Fas-mediated cytotoxicity, as illustrated by the devastating hepatic injury with massive hepatocyte apoptosis and eventual death of the animals triggered in a few hours by agonistic anti-Fas antibody (aFas) or FasL in mice.^{13,14} This model has been largely used as an experimental equivalent of fulminant human hepatitis.

TNF α (TNFSF2) is a pleiotropic cytokine that can promote such diverse effects as cell proliferation, differentiation, survival, or death, being involved in a variety of physiological and pathological processes. Two different receptors, p55 TNFR1 (TNFRSF1A) and p75 TNFR2 (TNFRSF1B), transduce the TNF α signal. TNFR1 is a death receptor whose DD upon ligand binding and receptor oligomerization recruits the adapter protein TRADD that provides docking sites for FADD and procaspase-8; the apoptotic cascade then progresses along the extrinsic or, frequently, the intrinsic pathway as reported above (reviewed by Ledgerwood *et al.*¹⁵). In addition, TNFR1 also transduces survival signals resulting in activation and nuclear translocation of the transcription factor NF- κ B, or in activation of members of the MAPK cascade.² In fact, TNF α may act as a double-edged sword by virtue of the multiplicity of signals it evokes, the final outcome of which will depend on

their balance as well as on the nature and recent history of the target cells.

The liver provides a well-known example for the dichotomy between trophic/mitogenic and cytotoxic effects of TNF α . A sound body of evidence substantiates the notion that TNF α can trigger hepatocyte proliferation *in vitro*^{16,17} as well as *in vivo* (reviewed by Michalopoulos and DeFrances¹⁸), and that normal hepatocytes are inherently resistant to TNF α toxicity. Thus, TNF α promotes regenerative liver growth, subsequent to partial hepatectomy¹⁹ or liver injury²⁰, and growth elicited by agents such as gadolinium chloride²¹ or peroxisome proliferators.¹⁶ On the other side of the coin, TNF α has been involved in the induction of cell death in experimental hepatitis^{22,23} and various liver diseases.²⁴ Moreover, its action on normal liver or primary hepatocyte cultures can be shifted to cytotoxicity by suppressing NF- κ B activation^{17,25–27} or by blocking macromolecular synthesis with translational/transcriptional inhibitors such as cycloheximide, actinomycin D, and galactosamine,²⁸ whose effect is attributed, in part at least, to inhibition of NF- κ B-regulated gene expression.^{26,27}

In view of (i) the above opposing actions of TNF α on hepatocytes, (ii) the notion that the cytotoxic TNFR1 and Fas signals converge onto the same FADD adapter and apoptotic cascades, and (iii) the hypothesis that endogenous TNF α might play a role in Fas-mediated liver injury,^{29,30} in the present study we compared the effects of a single dose of aFas in normal mice (R⁺) *versus* mice (R⁻) knocked out for both TNFR1 and TNFR2.²⁰ The results neatly show that, while R⁺ mice die within a few hours after challenge with aFas and develop a massive liver destruction, as originally shown by Ogasawara *et al.*,¹⁴ R⁻ mice are still alive at 24 h and their liver substantially spared from injury. These findings strongly argue for TNF α as being critically involved in the fulminant liver damage and animal death triggered by Fas activation, although the precise cellular and molecular basis for the synergy between Fas and TNFRs signaling largely remain an open question.

Results

In a matter of hours, a single i.v. injection of aFas into mice is known to cause both a devastating liver injury, resembling fulminant hepatitis, and animal death, regarded as an aftermath of the liver damage.¹⁴ Consistently, in the present study R⁺ mice died within 5–8 h after an i.v. dose of 10 μ g aFas. By contrast, all R⁻ animals were alive and looked healthy when killed, 10 or 24 h after treatment (Figure 1). At necropsy, the liver was swollen and highly congested in R⁺ animals, but had a virtually normal appearance in the R⁻ group.

Histologically, the aFas-induced liver injury drastically differed in severity between the two groups of animals. In R⁺ mice, massive panlobular apoptosis and diffuse hemorrhage (*peliosis hepatis*) were prominent, while the hepatic microarchitecture was no longer discernible (Figure 2a). The tissue was crowded with apoptotic bodies, either still containing nuclear material or showing as acidophilic (Councilman) bodies, that were interspersed with scarce hepatocytes still recognizable (Figure 2a), usually exhibiting a grossly vacuolated cytoplasm. Electron microscopy confirmed the occur-

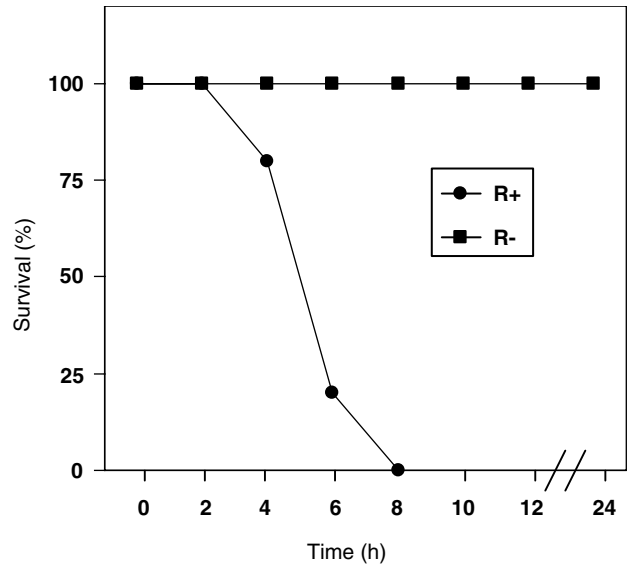


Figure 1 Resistance of R⁻ mice to the lethal effect of aFas. Percent survival of wild-type (●) and TNFRs-null (■) animals in response to a single i.v. dose of aFas ($n=10$)

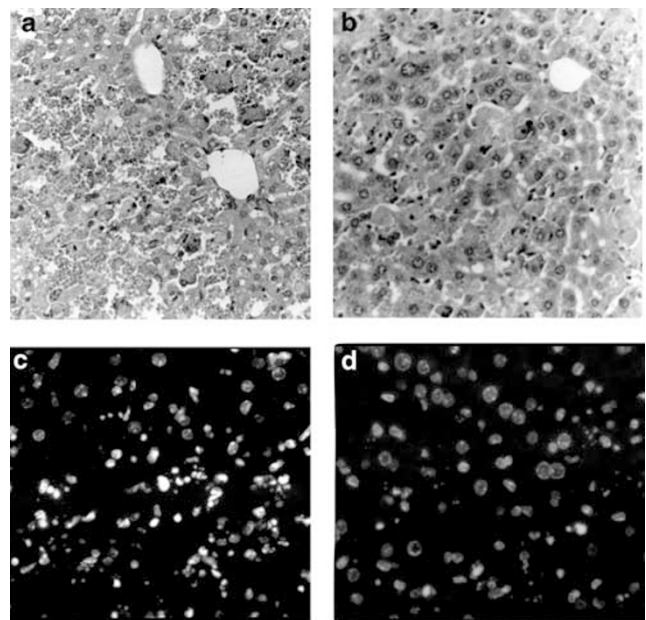


Figure 2 Histological appearance of the liver from R⁺ and R⁻ mice treated with aFas. Hematoxylin and eosin: R⁺ (a), R⁻ (b), magnification $\times 640$. DAPI: R⁺ (c), R⁻ (d), magnification $\times 640$

rence of extensive apoptosis, with a number of nuclei that showed the typical apoptotic morphology characterized by chromatin condensation, nucleolar segregation, and nuclear fragmentation (Figure 3a), while cytoplasmic organelles were relatively well preserved. Frequently, in the widespread areas of hemorrhage, dead cells appeared to float free among extravasated erythrocytes (Figure 3b). By contrast, in R⁻ mice, the lobular design and the characteristic radial distribution of hepatic trabeculae around the centrilobular vein were preserved, although in some animals discrete hemorrhagic

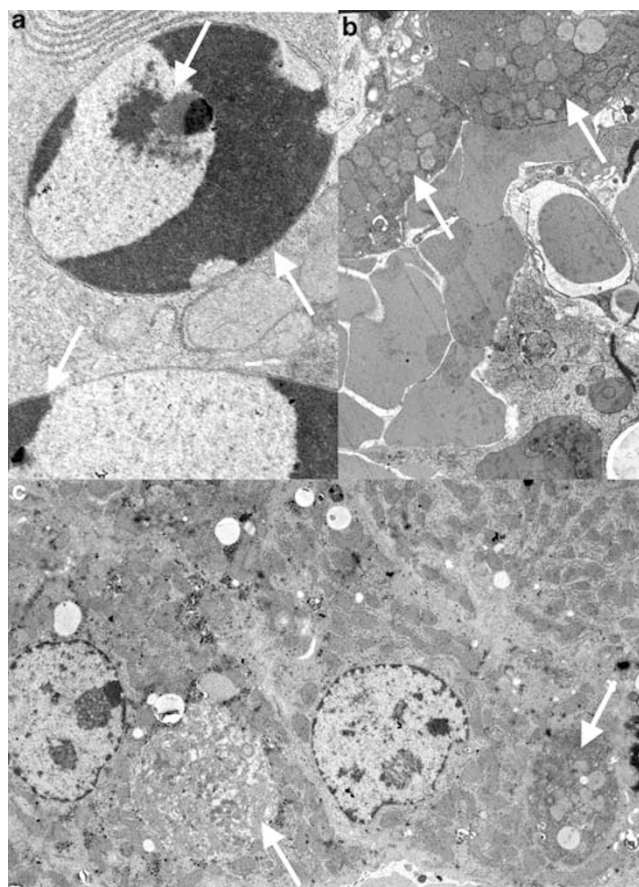


Figure 3 Transmission electron microscopy of the liver after aFas treatment. Panels a and b (magnification: $\times 12\,000$), R^+ animals; panel a details the nuclear alteration: chromatin condensation and nucleolar segregation (arrows); panel b shows some apoptotic bodies containing well recognizable cytoplasmic organelles (arrows). Panel c (magnification $\times 7100$) shows that the liver of R^- mice is comparatively well preserved; the arrows indicate huge intracellular vacuoles, likely deriving from phagocytosis of apoptotic bodies

foci could be detected (Figure 2b). The number of apoptotic cells in R^- livers, although quite remarkable compared to untreated animals, was drastically lower than in R^+ animals with dead cells distributed throughout the lobular zones, while most hepatocytes showed a marked cytoplasmic vacuolation (Figure 2b). Electron microscopy confirms that most hepatocytes show a fairly well-preserved morphology. Some of them contain huge vacuolar structures likely representing phagocytosed apoptotic bodies (Figure 3c). The marked difference between the two groups of mice in the frequency of apoptotic nuclei, showing condensed or marginated chromatin or frankly fragmented, is easily appreciated in preparations stained with the DAPI fluorochrome (Figures 2c and d).

Internucleosomal DNA fragmentation is an established, albeit not universal, hallmark of apoptotic cell death. Most DNA extracted from aFas-treated R^+ mice liver indeed appeared extensively degraded such as to produce a prominent 'ladder pattern' by agarose-gel electrophoresis, while DNA fragmentation, although still detectable, was much less pronounced in R^- animals (Figure 4).

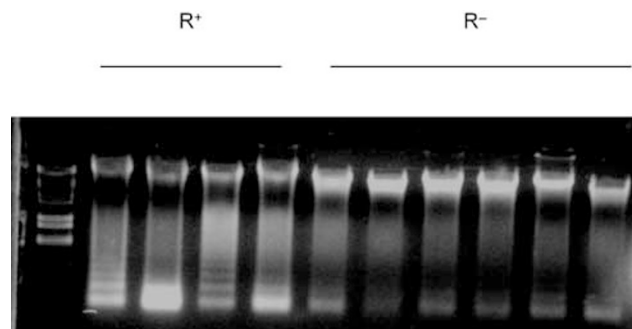


Figure 4 Agarose gel electrophoresis of DNA extracted from the liver of R^+ and R^- animals treated with aFas (5–8 h for R^+ and 10 h for R^-). Lane 1: molecular weight markers, lanes 2–5: R^+ mice; lanes 6–11: R^- mice

The induction of apoptosis typically requires activation of both initiator and effector caspases. Cotreatment with the pancaspase inhibitors z-VAD-fmk³¹ or IDN-1965³² indeed protects mice from aFas-induced liver injury. In the present experiments, caspase 3-, 8-, and 9-like activities were definitely lower in R^- than in R^+ mice livers (Table 1), consistently with the different extent of apoptosis. Other proteolytic enzymes have been implicated in the apoptotic process: calpains have been shown to activate the caspase cascade in both B and T cells, to cleave the proapoptotic factor Bid, and to be involved in microcystin-induced apoptosis in isolated rat hepatocytes.^{33–36} Moreover, the lysosomal cathepsins B and D have been shown to play a role in TNF α -induced apoptosis of various cells, hepatocytes included.^{37–39} In the present study, both cathepsin B and, particularly, calpain activities were significantly higher in R^+ than in R^- animals treated with aFas (Table 1). Therefore, diverse proteolytic systems relevant to apoptosis were all more active in R^+ mice livers at a stage in which aFas-induced injury was fully developed, while in R^- mice these proteolytic systems were much less active at the same time interval after aFas administration, further confirming that the lack of TNFRs confers resistance to aFas.

An obvious possible explanation for the different susceptibility of R^- versus R^+ animals to Fas-induced liver injury is that abrogation of 'tonic' signaling from TNFRs may somehow downregulate Fas expression in the liver.⁴⁰ By Western blotting, neither Fas nor FasL protein levels significantly differed between the two mice groups after aFas administration (Figure 5a, b). In addition, untreated R^+ and R^- animals did not differ as to cell surface distribution of Fas (Figure 5c) and total Fas protein levels (data not shown).

Hepatocytes can be categorized as type II cells in that execution of Fas-mediated apoptosis requires activation of the 'intrinsic' or mitochondrial pathway (see Introduction). The latter is extensively modulated by pro- and antiapoptotic members of the Bcl-2 family. Consistently, expression of a Bcl-2 transgene largely protects mice from aFas hepatic toxicity,^{41,42} although not necessarily from lethality,⁴² while increased hepatic Bcl-X_L expression would account for the higher resistance to aFas of C/EBP knockout mice.⁴³ In the present work, we examined the level of the antiapoptotic Bcl-2 protein. Although not different in untreated animals, Bcl-2

Table 1 Enzymatic activities in the liver of R⁺ and R⁻ mice after treatment with aFas

	Controls		Treated with aFas	
	R ⁺ (5)	R ⁻ (5)	R ⁺ (8)	R ⁻ (8)
Caspase 3	16.0 ± 4.5	21.0 ± 9.4	66.9 ± 9.0*	23.3 ± 3.2**
Caspase 8	27.2 ± 9.0	34.0 ± 8.1	88.1 ± 13.6*	31.3 ± 9.4**
Caspase 9	21.4 ± 11.1	27.9 ± 16.1	139.2 ± 34.0*	34.2 ± 9.8**
Calpain	19.7 ± 6.3	19.0 ± 7.0	104.9 ± 18.5*	29.5 ± 7.9**
Cathepsin B	10.2 ± 3.1	10.9 ± 1.1	24.4 ± 4.0*	10.6 ± 4.4**

Data (nkatal × 10⁻⁵/mg protein) expressed as means ± SD. No. of animals in brackets. The liver from R⁻ mice has been collected between 5 and 8 h postinjection (immediately before the death of each animal), while R⁺ mice were killed 10 h after anti-Fas administration (see Materials and Methods). Significance of the differences (ANOVA): **P* versus untreated R⁺; ***P* versus treated R⁺.

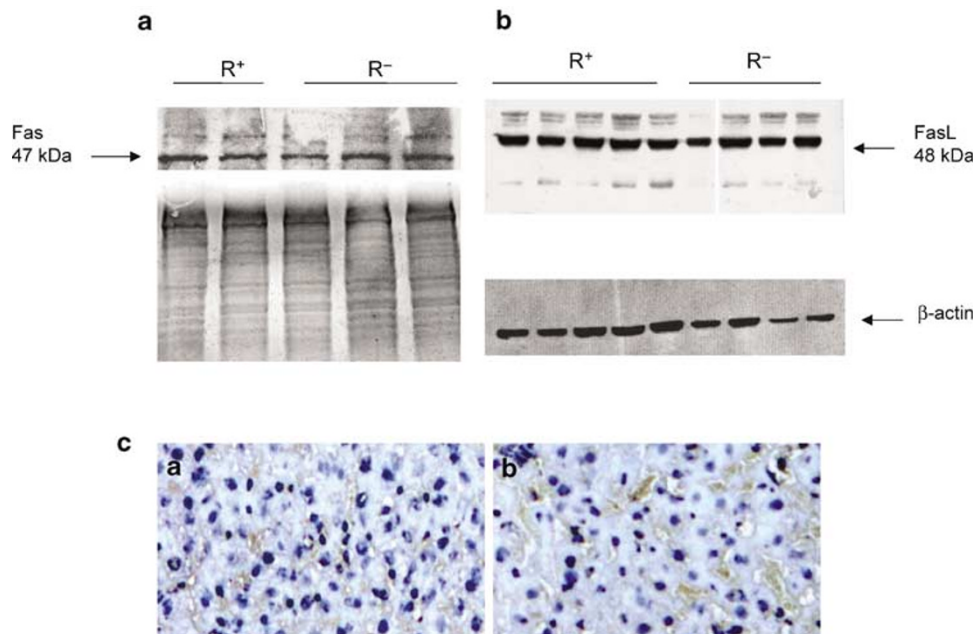


Figure 5 Fas and FasL expression in the liver of R⁺ and R⁻ mice. (a) Western blotting indicating Fas expression in aFas-treated mice; R⁺ (lanes 1–2), R⁻ (lanes 3–5); (b) Western blotting analysis of FasL expression in aFas-treated animals, R⁻ (lanes 1–5), R⁺ (lanes 6–9); homogeneity of protein loading checked by Coomassie blue staining (Fas) or β -actin expression (FasL). Time after aFas treatment: 5–8 h for R⁺ and 10 h for R⁻. (c) Immunohistochemical analysis of Fas distribution in untreated R⁺ (a) and R⁻ (b) mice

markedly increased after aFas treatment in the liver of R⁻ mice only (Figure 6), raising the possibility that this response may contribute to their lower susceptibility to Fas engagement.

Finally, as a measure of its activation status, we evaluated the DNA-binding activity of the transcription factor NF- κ B, which is known to modulate the cell response to death receptor signals and various other cellular stresses. Figure 7 shows that the DNA-binding activity of NF- κ B, already detectable in ¹R⁻, though less than in ²R⁻ controls, was enhanced moderately in R⁺ mice and markedly in R⁻ mice after aFas treatment. Consistently with this result, the levels of the I- κ B inhibitory subunit are reduced in both R⁺- and R⁻-treated animals, although no clear differences can be observed between the two groups (R⁺:13 ± 3, R⁻:22 ± 4 densitometric units). This lack of difference may depend from an increased I- κ B production in the R⁻ mice after aFas

treatment, a well-known consequence of NF- κ B activation. R⁻ animals are characterized by a higher basal activity of NF- κ B, which does not associate with modulations in the levels of the inhibitory subunit I- κ B (30 ± 4 and 38 ± 6 densitometric units in R⁺ and R⁻ mice, respectively). Moreover, in these animals, aFas treatment results in a much higher activation of this transcription factor. This differential may also concur to the higher resistance of R⁻ mice to aFas.

Discussion

The present paper shows that mice lacking both TNFR1 and TNFR2 (R⁻) are resistant to a single dose of aFas, which is lethal for TNFR-expressing animals and that their liver is only mildly affected, compared to the devastating hepatic damage that in a few hours strikes wild-type mice. R⁻ animals show no

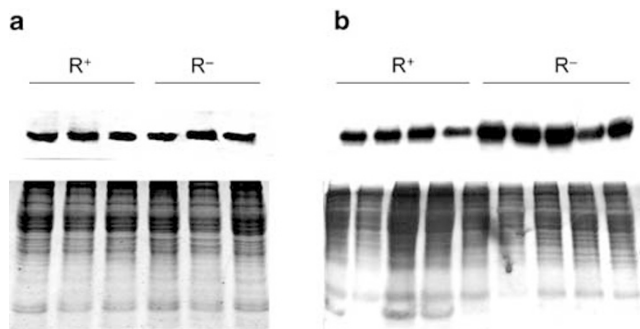


Figure 6 Western blots of Bcl-2 in the liver of R⁺ and R⁻ mice before (a) and after (b) treatment with aFas (5–8 h for R⁺ and 10 h for R⁻). (a) R⁺ (lanes 1–3), R⁻ (lanes 4–6), (b) R⁺ (lanes 1–4), R⁻ (lanes 5–9); homogeneity of protein loading checked by Coomassie Blue staining

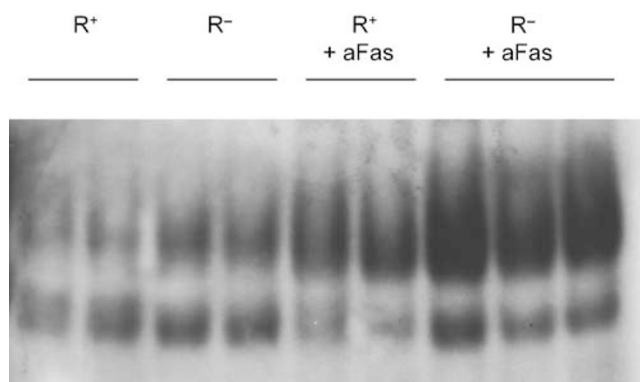


Figure 7 EMSA analysis of the DNA-ligand binding activity of NF- κ B. Lanes 1–2 (untreated R⁺), 3–4 (untreated R⁻), 5–6 (aFas-treated R⁺ 5–8 h postinjection), 7–9 (aFas-treated R⁻, 10 h postinjection)

alteration in the overall hepatic levels or the cell surface expression of Fas. A marked upregulation of Bcl2 expression occurs in R⁻ though not in R⁺ mice after challenge with aFas. In addition, the DNA-binding activity of the NF- κ B transcription factor, already slightly higher in R⁻ than in R⁺ control animals, after aFas treatment is enhanced much more markedly in R⁻ than in R⁺ mice. Therefore, the present evidence consistently demonstrates that a normal TNFR1/TNFR2 phenotype is required for full deployment of the aFas toxicity. These observations thus strongly suggest that treatment with aFas activates the TNF α -TNFRs system, whose signaling, in synergy with that generated by Fas, appears to be required for fulminant liver injury and animal death to develop.

Protection against aFas toxicity has been afforded by various means, including pancaspase inhibitors^{31,32} or high tissue levels of Bcl-2 or Bcl-X_L.^{41–43} Treatment with an IL15-IgG2b fusion protein is also protective²⁹ and activated IL15R α has been shown to bind TRAF2,³⁰ which is a major signal transducer for TNF α as well as other cytokines and mostly plays a cytoprotective role, particularly via NF- κ B activation (reviewed by Chung *et al.*⁴⁴). Evidence that may specifically support a synergic role for endogenous TNF α in aFas toxicity has been provided by a recent report⁴⁵ showing that administration of aFas causes an increase of TNF α plasma levels and that partial protection from aFas-induced liver injury

can be afforded by pretreating mice with gadolinium chloride, an agent that temporarily depletes the large periportal Kupffer cells. This observation led to depict Fas-mediated toxicity as a two-step process in which Fas causes Kupffer cells to release TNF α that in turn promotes hepatocyte death.⁴⁵ In principle, this view is compatible with the present findings. However, in addition to Kupffer cell depletion, gadolinium chloride exerts on the liver other effects potentially relevant to the protection from aFas, such as a marked increase in TNF α mRNA levels, NF- κ B activation or even stimulation of hepatocyte proliferation; of interest, all of these effects are suppressed by anti-TNF α antibodies.^{21,46,47}

Protection against aFas-mediated toxicity has also been observed in the regenerating liver. Indeed, mice at 24 h after two-thirds hepatectomy are resistant to aFas-induced toxicity,^{1,19} although the hepatocyte surface expression of Fas is unchanged.¹⁹ On the other hand, sublethal doses of aFas afford protection against a subsequent lethal challenge with the same antibody, an effect that has been ascribed to the compensatory proliferation elicited by the first injection.¹ In this context, the proliferation-promoting role of TNF α in the liver seems to be involved in the resistance to aFas-induced damage. Indeed, exogenous TNF α given 5 h before aFas in nonoperated animals and anti-TNF α antibodies given prior to hepatectomy, respectively, reduce and increase the susceptibility to aFas.¹⁹ Noteworthy, aFas itself accelerates the compensatory liver growth after partial hepatectomy.¹ Altogether, the notion clearly emerges that, whatever their precise nature, mechanisms are activated in the regenerating liver to suppress not only TNFRs- but also Fas-mediated death signals, in contrast to their apparent cooperation in actuating aFas toxicity in the normal liver.

The present work does not discriminate whether TNFRs relay to hepatocytes stimuli from nonparenchymal cells, such as sinusoidal endothelial³¹ or Kupffer cells,⁴⁵ or rather their signals integrate with those generated by Fas directly within the same cells targeted by aFas. Early studies already reported that surface TNFRs are internalized in Jurkat T cells undergoing apoptosis by aFas,⁴⁸ while a recent report shows that TNFR2-null CD8 T cells are resistant to aFas treatment.⁴⁹ Moreover, potential ‘crosstalks’ between the TNF α -TNFRs and FasL-Fas signaling pathways have been hypothesized in view of the shared role of FADD^{50,51} and of other commonalities such as positive and negative regulators of the apoptotic cascades, JNK activation, and modulation of transcription factors such as NF- κ B or AP-1.

Finally, the R⁻ phenotype might render liver cells inherently more resistant to aFas by modifying the expression profile or regulation of relevant gene products. The present work provides two relevant examples in this regard. Bcl-2 expression, comparable in untreated R⁺ and R⁻ animals, after aFas treatment was upregulated in R⁻ mice only, suggesting the obvious speculation that in R⁺ mice this upregulation might be prevented by the Fas-activated TNFRs signalling. TNF α has indeed been reported to suppress Bcl-2 expression in hepatoma⁵² and microglial cells.⁴⁰ Furthermore, the DNA-binding activity of NF- κ B, already slightly higher in R⁻ than R⁺ controls, after aFas treatment was much more strongly activated in R⁻ than in R⁺ mice. Although the underlying mechanisms require further investigations, it seems concei-

vable that these regulations may both concur to reduce the susceptibility of R⁻ mice to aFas.

Materials and Methods

Animals and treatment

The experiments have been performed using 8-week-old C57BL6 mice knocked out for both TNFRs (R⁻ mice), kindly provided by Dr. H Bluethmann, Hoffmann-La Roche Ltd, Basel, Switzerland. Homozygous *tnfr1/tnfr2* double knockout mice (R⁻ mice) were generated by crossbreeding *tnfr1*^{-/-} and *tnfr2*^{-/-} mice.⁵³ C57BL6 mice (Charles River, Calco, Italy) have been used as controls (R⁺ mice). The animals have been housed and cared for in compliance with the Italian Ministry of Health Guidelines (n. 86/609/EEC) and with the Principles of Laboratory Animal Care (NIH n. 85-23, revised 1985).

Both R⁺ and R⁻ mice, weighing approximately 25–30 g, received intravenously 10 µg anti-Fas antibody (Jo2, Pharmingen, San Diego, CA, USA). R⁺ mice became moribund within 5–8 h after treatment. Some of them were immediately killed under anesthesia with zoletil, while some others have been monitored to evaluate the exact time of death. A group of R⁻ mice was killed 10 h after the injection of aFas, while some animals were monitored for the following 24 h to analyze survival. The liver was rapidly excised, partly frozen in liquid nitrogen and stored at -80°C, partly fixed in 4% (w/v) paraformaldehyde or in 4% (v/v) Karnowski solution (25% paraformaldehyde, 25% glutaraldehyde, 0.1 M cacodylate buffer, pH 7.3) and stored at 4°C.

Morphological analysis

- Light microscopy
Different liver lobes previously fixed in paraformaldehyde were routinely embedded in paraffin. Slices 5 µm thick were stained with hematoxylin and eosin. To better visualize chromatin, some slices were stained with DAPI (10 ng/ml in methanol), washed with PBS followed by absolute ethanol, mounted in Bacto-Fa (Difco, USA), and viewed in an epiilluminated fluorescence microscope (Dialux 20, Leitz, Germany).
- Electron microscopy
Tissues fixed in Karnowski solution were postfixed with 1% (w/v) osmium tetroxide, dehydrated with sequential passages in an acetone ascending series (50–70–90–100%), and embedded in Epon. Gray-silver ultrathin sections (4 nm), mounted on copper grids, were stained with uranyl acetate–lead nitrate, and examined with a Zeiss EM10 electron microscope.
- Immunohistochemistry
Tissues previously frozen in liquid nitrogen were embedded in O.C.T. compound (Miles, Elkhart, IN, USA). Slices 5 µm thick (Leitz 1720 cryostat) were methanol-fixed, stained with anti-Fas primary antibody (1 : 50 overnight; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and incubated with biotinylated goat anti-rabbit secondary antibody and streptavidin (LSAB2 kit, Dako, Milano, Italy). The reaction was developed with diaminobenzidine and H₂O₂ as chromogenic substrate.

DNA fragmentation

The occurrence of internucleosomal DNA fragmentation was evaluated by agarose gel electrophoresis as described by Kaufmann.⁵⁴ The liver was homogenized with a Polytron apparatus in a lysis buffer (2 mM EDTA, 10 mM NaCl, 1% SDS, 500 mM TRIS-HCl, pH 9.0), the homogenate

incubated overnight at 48°C in the presence of proteinase K (20 mg/ml) and DNA was extracted with the phenol–chloroform–isoamyl alcohol standard procedure. The resulting aqueous phase was incubated 60 min in the presence of 10 mg/ml RNAse at 37°C. Aliquots of 20 µg DNA were electrophoresed on 1% agarose gel and viewed under UV illumination after staining with ethidium bromide.

Western blotting

The liver was homogenized in 80 mM TRIS-HCl, pH 6.8, containing 0.1 M DTT, 70 mM SDS, and 1 mM glycerol, kept in ice for 30 min, centrifuged at 15 000 × g for 10 min at 4°C, and the supernatants collected. Protein concentration was determined by the method of Lowry *et al.*,⁵⁵ using BSA as working standard. Equal amounts of proteins (50 µg) were heat-denatured in sample-loading buffer (50 mM TRIS-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol), resolved by SDS-polyacrylamide gel electrophoresis (10% polyacrylamide, 0.1% SDS) and transferred to nitrocellulose membranes (Hybond C, Amersham Italia, Cologno Monzese, Milano, Italy). The filters were blocked with 5% PBS-BSA at 4°C overnight, then incubated with polyclonal antibodies directed against Bcl-2, Fas, FasL, and I-κBα (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad, Hercules, CA, USA) was used as secondary antibody. The membrane-bound immune complexes were detected by an enhanced chemiluminescence system (NEN, Boston, MA, USA).

Enzymatic activities

Liver samples were homogenized in 20 mM HEPES-KOH, pH 7.5, containing 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, frozen–thawed, sonicated and centrifuged (13 000 r.p.m., 15 min, 4°C). Aliquots of the supernatant corresponding to 10 µg protein were diluted in 25 mM HEPES pH 7.5, containing 0.1% CHAPS, 10% sucrose, 10 mM DTT, and assayed for caspase 3-, 8-, and 9-like, calpain and cathepsin B activities in the presence of the corresponding substrates (caspase 3: DEVD-AMC; caspase 8: IETD-AMC, caspase 9: LEHD-AMC; calpain: Suc-Leu-Tyr-AMC; cathepsin B: Z-Arg-Arg-AMC; Sigma). After 1 h incubation at 37°C, the reaction was stopped with 0.1% TCA and fluorescence was read in a Perkin-Elmer fluorometer (excitation 380 nm, emission 460 nm). Free AMC was used as standard.

Electrophoretic mobility shift assay

Nuclear extracts from the liver have been obtained as described by Xu and *et al.*¹⁷ Briefly, the tissue has been homogenized in 20 mM HEPES, pH 7.9, containing 350 mM NaCl, 1 mM MgCl₂, 0.5 mM EDTA, 0.1 mM EGTA, 20% glycerol, 1% Nonidet P-40, 0.1% PMSF, 0.5 mM DTT, 10 µg/ml leupeptin, and the nuclei isolated by centrifugation. Isolated nuclear fractions were lysed in a 1 : 1 mixture of the above buffer and another one containing 25 mM HEPES, pH 7.6, 0.1 mM EDTA, 20% glycerol, 1 mM PMSF, 1 mM DTT, 10 µg/ml leupeptin, and stored at -80°C until used for assay.

Oligonucleotide labeling and binding reactions were performed by using the reagent supplied in the Gel Shift Assay System (Promega Italia, Milano, Italy). Nuclear proteins (10 µg) were incubated at room temperature for 20 min in the presence of the ³²P-ATP end-labeled, double-stranded NF-κB consensus oligonucleotide (Promega Italia, Milano, Italy), as directed by the manufacturer. At the end of the incubation, 0.1 vol of 10 × gel loading buffer (250 mM Tris-HCl, pH 7.5, 0.2% bromophenol blue, 40% glycerol) was added to the binding mixture

and the samples electrophoresed in $0.5 \times$ TBE buffer at 350 V for 40 min on a 4% nondenaturing acrylamide gel. After electrophoresis, the gel was dried for 30 min in a BioRad Gel Dryer and exposed overnight or for longer times to an X-ray sensitive film (Hyperfilm-MP, Amersham Biosciences, Cologno Monzese, Milano, Italy) at -80°C with intensifying screens. The specificity of the bands has been confirmed by adding an excess amount of cold oligonucleotide to the reaction mixture (not shown).

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