# Caspase-1 is not involved in experimental hepatitis in mouse

Wim van Molle, Peter Brouckaert, Claude Libert*

*Department of Molecular Biology, Flanders Interuniversity Institute for Biotechnology and University of Ghent, K.L. Ledeganckstraat 35, B-9000 Ghent, Belgium*

Received 17 December 1998; received in revised form 18 January 1999

**Abstract Experimental hepatitis induced by tumor necrosis factor in D-(+)-galactosamine-sensitized mice or by an agonistic anti-Fas antibody in normal mice is accompanied by dramatic apoptosis of hepatocytes. Apoptosis is the final result of activation of a cascade of caspases. We used caspase-$1^{-/-}$ mice, generated by gene targeting, to study the role of this protease in TNF- and anti-Fas-induced lethal hepatitis. We found that mutant mice exhibited the typical caspase-$1^{-/-}$ phenotype, since they resisted to a lethal injection of LPS and released no interleukin-1β in the circulation, in contrast to wild-type littermates. When caspase-$1^{-/-}$ mice were challenged with different doses of tumor necrosis factor/D-(+)-galactosamine or with anti-Fas, no increased survival was observed compared with control mice. Furthermore, apoptosis in the livers of these mice and serum levels of alanine aminotransferase were not reduced. These data indicate that caspase-1 deficiency does not lead to reduced apoptosis in these models, either because caspase-1 is irrelevant in this model or because of functional redundancy.**



*Key words:* Apoptosis; Caspase; Hepatitis; Tumor necrosis factor

## 1. Introduction

Induction of liver failure by tumor necrosis factor (TNF) has been recognized as being a major dose-limiting toxicity, hampering the use of TNF as an antitumor drug. Several model systems have been developed to study the mechanism by which TNF induces necrosis of the liver. It was found that TNF injection in mice sensitized by D-(+)-galactosamine (GalN) leads to a specific lethal hepatitis, involving massive induction of apoptosis of hepatocytes [1,2] and which resembles very closely the lethal hepatitis induced by triggering Fas/CD95/APO-1 [3], a member of the TNF-receptor superfamily.

Apoptosis has been found to be the result of cleavage of essential substrates and chromosomal DNA by activated caspases, which are aspartate-specific proteases [4]. Caspase-1 or homologues were found to be involved in TNF- and anti-Fas-induced apoptosis and to activate other caspases [5]. Recently, two groups independently described the generation of caspase-$1^{-/-}$ mice by gene targeting [6,7]. These mice had developed normally, which is surprising since apoptosis is an essential event during the development of, for example, the extremities. A reduced response of T-cells to anti-Fas-induced apoptosis was found [6] along with a resistance to an otherwise lethal injection of LPS [7]. Recently, it was shown that caspase-1 is responsible for activating interferon-γ inducing factor, also known as interleukin-18, and that caspase-$1^{-/-}$ mice fail to produce interferon-γ after stimulation with LPS [8].

We were interested to know to what extent caspase-1 is involved in the induction of apoptosis in the liver, after a challenge with TNF or anti-Fas. We used caspase-$1^{-/-}$ mice and studied the survival, metabolic parameters and death as an endpoint. We conclude that the absence of caspase-1 does not lead to reduced apoptosis in both the TNF/GalN and the anti-Fas model.

## 2. Materials and methods

### 2.1. Animals

The female caspase-$1^{-/-}$ mice [7] and control animals were a kind gift of Dr. T. Seshadri (BASF Bioresearch Corporation, Worcester, MA, USA) and were bred as homozygotes at our facilities. Mice were used at the age of 8–15 weeks. The animals were housed in a temperature-controlled, air-conditioned room with 12 h light/dark cycles, and received water and food ad libitum.

### 2.2. Reagents

Murine TNF (mTNF), produced and purified to homogeneity in our laboratory, had a specific activity of $2.3\times10^8$ IU/mg: the endotoxin contamination was 0.2 ng/mg protein. The endotoxin levels were assessed with a chromogenic Limulus amoebocyte lysate assay (Coatest: Chromogenix, Stockholm, Sweden). LPS (*E. coli* O111:B4) was purchased from Difco Laboratories (Detroit, MI, USA), GalN was obtained from Sigma Chemical (St. Louis, MO, USA). Agonistic monoclonal hamster anti-mouse Fas was purchased from PharMingen (San Diego, CA, USA) and had an endotoxin level of 0.05 ng/mg protein, according to the manufacturer.

### 2.3. Injections and blood collections

Reagents were diluted in LPS-free PBS before use. Intraperitoneal (i.p.) injections had a volume of 0.5 ml and intravenous (i.v.) injections were given in a volume of 0.2 ml. Blood for alanine aminotransferase (ALT) was taken from the retro-orbital plexus under ether anesthesia. Serum was prepared by allowing blood to clot for 30 min at 37°C and 1 h at 4°C, followed by centrifugation at $16\,000\times g$.

### 2.4. Assays

Serum ALT was determined using an enzymatic/colorimetric ALT kit (Sigma Chemical). Interleukin-1β (IL-1β) concentrations in the serum were estimated using a D10 bioassay [9].

### 2.5. Tests for apoptosis

Apoptosis was quantified in a cell death detection ELISA kit (Boehringher Mannheim, Mannheim, Germany). Before excision, livers were perfused for 10 s with perfusion buffer (50 mM phosphate, 120 mM NaCl, 10 mM EDTA, pH 7.4). A 20% homogenate (w/w) was made in the same buffer with a homogenizer (Heidolph-Elektro, Kelheim, Germany: model RZR 2020 at position II-10) and centrifuged for 20 min at $13\,000\times g$. The supernatant of approximately 25 μg of liver was used in the ELISA as described previously [2]. Internucleosomal DNA cleavage was confirmed by gel electrophoresis. DNA was prepared from 500 μl of liver homogenate. The proteins were removed once with phenol/chloroform/isoamylalcohol (50/48/2) and once with chloroform/isoamylalcohol (24/1). DNA was precipitated overnight with 5 M ammoniumacetate and ethanol at −20°C. DNA was resolved in Tris/EDTA and RNA was removed by RNase-

*Corresponding author. Fax: (32) (9) 264 53 04.
E-mail: claude@dmb.rug.ac.be


PII: S0014-5793(99)00109-X

Table 1
Sensitivity of wt and caspase-$1^{-/-}$ mice, and induction of IL-1β after a lethal challenge with LPS

| Challenge | Mice | IL-1β (pg/ml) | Lethality |
|---|---|---|---|
| 800 μg LPS i.p. | Caspase-$1^{+/+}$ | 474 ± 208 | 6/6 |
| 800 μg LPS i.p. | Caspase-$1^{-/-}$ | Not detectable | 0/6 |

IL-1β (mean ± S.D.) was measured in the serum, collected 4 h after the challenge. Lethality (deaths/total) was scored 72 h after the challenge (no further deaths occurred).

H treatment. The samples were then analyzed by gel electrophoresis in a 1.8% agarose gel, followed by ethidium bromide staining.

*2.6. Statistics*

Mean values (and S.D.) were compared using an unpaired Student's *t*-test, with Welch's correction in the case of non-homogeneous variances. Survival curves (Kaplan-Meier plots) were compared using a $chi^2$ test.

## 3. Results

*3.1. Caspase-$1^{-/-}$ mice are resistant to a lethal challenge with LPS and produce no mature IL-1β*

It was reported that caspase-$1^{-/-}$ mice are resistant to a systemic inflammatory response syndrome induced by LPS [7]. Furthermore, these mice do not produce mature IL-1β. In our first experiment, we intended to repeat these findings. Therefore caspase-$1^{-/-}$ mice and wild-type (wt) control mice were challenged i.p. with 800 μg LPS. This dose and LPS type are identical as in the original paper [7]. 4 h After the challenge, blood was withdrawn for IL-1β determinations and lethality was assessed up to 72 h after the challenge. In Table 1 we demonstrate that in contrast to wt mice, caspase-$1^{-/-}$ mice produced no detectable IL-1β. Furthermore, all wt mice died to the challenge while all caspase-$1^{-/-}$ mice survived and displayed no signs of distress. This experiment confirmed the results obtained by Li et al. [7].

*3.2. Caspase-1 is not essential for the lethality induced by TNF+GalN or anti-Fas*

We and others described that TNF+GalN causes massive in vivo apoptosis of hepatocytes in mice [2,10]. Similar observations were also made after a challenge with an agonistic anti-Fas antibody [2,3]. To test the possible in vivo role of caspase-1, caspase-$1^{-/-}$ mice and wt control mice were challenged i.p. with 20 mg GalN in combination with different doses of mTNF, 0.1 and 0.5 μg. The survival was assessed up to 48 h after the challenge. Fig. 1 demonstrates that 0.1 μg is a sublethal dose, both for wt and caspase-$1^{-/-}$ mice. No difference in final lethality was recorded ($P > 0.05$) for the two doses. Wt and caspase-$1^{-/-}$ mice were treated i.v. with 5 or 15 μg anti-Fas. Lethality was followed up to 24 h after the challenge. In Fig. 2, we demonstrate that 5 μg anti-Fas causes partial lethality in both types of mice. 100% lethality is obtained with 15 μg anti-Fas. Also in this model, no significant differences between wt and caspase-$1^{-/-}$ mice were found ($P > 0.05$). These results demonstrate that the absence of caspase-1 has not led to a reduced lethal response induced either by TNF+GalN or by anti-Fas.

*3.3. The absence of caspase-1 does not prevent apoptosis induced by TNF+GalN or anti-Fas*

We were interested to know whether apoptosis can still occur in the hepatocytes since an in vitro involvement of caspase-1 in induction of apoptosis by TNF or anti-Fas had been described [11,12]. Caspase-$1^{-/-}$ and wt mice were challenged i.p. with 0.5 μg mTNF+20 mg GalN or i.v. with 15 μg anti-Fas. To check the absence of apoptosis, mice were also injected with 20 mg GalN or 0.5 μg mTNF. 5 h (anti-Fas) or 8 h (TNF/GalN) after the challenge, blood was collected for ALT measurements and mice were killed to remove livers for determination of DNA fragmentation. At the moment livers were excised, it was clear that necrosis had occurred both in

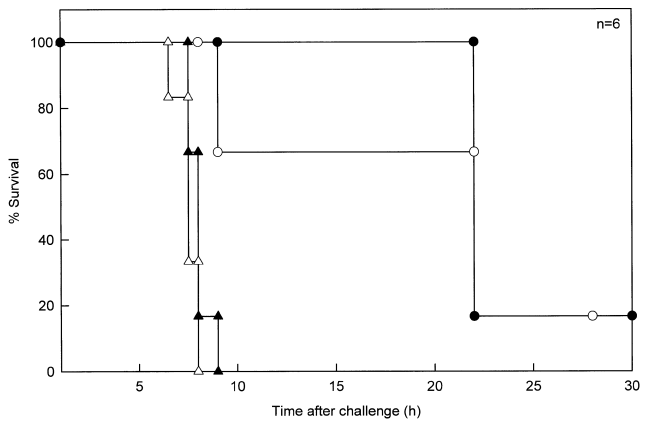


Fig. 1. Induction of lethality in wt and caspase-$1^{-/-}$ mice by TNF+GalN. Wt mice (open symbols) and caspase-$1^{-/-}$ mice (closed symbols) were challenged i.p. with 20 mg GalN in combination with 0.1 μg mTNF (○) or 0.5 μg mTNF (△). Lethality was evaluated up to 48 h. No further deaths occurred.

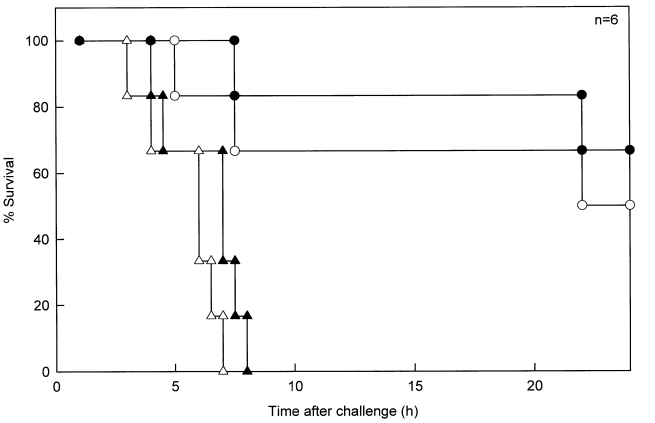


Fig. 2. Induction of lethality in wt and caspase-$1^{-/-}$ mice by anti-Fas. Wt mice (open symbols) and caspase-$1^{-/-}$ mice (closed symbols) were challenged i.v. with 5 μg anti-Fas (○) or 15 μg anti-Fas (△). Lethality was evaluated up to 48 h. No further deaths occurred.

Table 2
DNA fragmentation of hepatocytes and the release of ALT in wt and caspase-$1^{-/-}$ mice after a lethal challenge with TNF/GalN or anti-Fas

| Challenge | Mice | DNA Fragmentation | Serum ALT |
|---|---|---|---|
| PBS | Caspase-$1^{+/+}$ | 100 ± 7 | 32 ± 14 |
| 0.5 μg mTNF+20 mg GalN | Caspase-$1^{+/+}$ | 955 ± 54 | 5156 ± 4358 |
| 0.5 μg mTNF | Caspase-$1^{+/+}$ | 130 ± 50 | 47 ± 14 |
| 20 mg GalN | Caspase-$1^{+/+}$ | 176 ± 57 | 72 ± 27 |
| 15 μg anti-Fas | Caspase-$1^{+/+}$ | 1014 ± 32 | 4991 ± 1020 |
| PBS | Caspase-$1^{-/-}$ | 100 ± 9 | 41 ± 11 |
| 0.5 μg mTNF+20 mg GalN | Caspase-$1^{-/-}$ | 987 ± 39 | 4368 ± 3158 |
| 0.5 μg mTNF | Caspase-$1^{-/-}$ | 96 ± 19 | 52 ± 19 |
| 20 mg GalN | Caspase-$1^{-/-}$ | 174 ± 58 | 66 ± 19 |
| 15 μg anti-Fas | Caspase-$1^{-/-}$ | 997 ± 77 | 6363 ± 225 |

Mice ($n = 6$) were injected i.p., except for anti-Fas given i.v. Livers were excised and blood was withdrawn 5 h after anti-Fas and 8 h after TNF/GalN, TNF and GalN challenge. DNA fragmentation is expressed as percentage of the control, serum ALT as U/l.

wt and caspase-$1^{-/-}$ mice after the challenge with TNF/GalN or anti-Fas. Livers were completely black because of infiltration of erythrocytes. Table 2 illustrates that apoptosis occurred in hepatocytes after a challenge with TNF+GalN or anti-Fas, both in wt and caspase-$1^{-/-}$ mice. No apoptosis was detected after injection with GalN or mTNF. Table 2 also shows that no significant differences within treatment groups, between wt and caspase-$1^{-/-}$ mice were found ($P > 0.05$). This was expected because of visible liver destruction when livers were removed. Concerning the ALT levels, we found that TNF+GalN and anti-Fas induced a dramatic release of ALT in both types of mice. GalN and TNF alone induced no over-background levels. These results suggest that caspase-1 deficiency has no effect on the induction of apoptosis in both the TNF/GalN and the anti-Fas model.

### 3.4. *Caspase-$1^{-/-}$ mice still show the DNA ladder pattern after a challenge with TNF/GalN or anti-Fas*

A hallmark of apoptosis is the presence of DNA ladder patterns that originate from DNA cleavage by an endonuclease, which cleaves between the nucleosomes resulting in fragments of multimers of 180 bp. As a confirmation of the results obtained by cell death ELISA, DNA was isolated from the liver homogenates, obtained in the experiment of Table 2, and analyzed on gel electrophoresis as described in Section 2. Fig. 3 shows DNA ladders in mice treated with TNF+GalN or anti-Fas in caspase-$1^{-/-}$ mice, as well as in control mice. No ladder pattern was seen in mice treated with PBS, GalN or mTNF. The results of this experiment confirm the data obtained by ELISA.

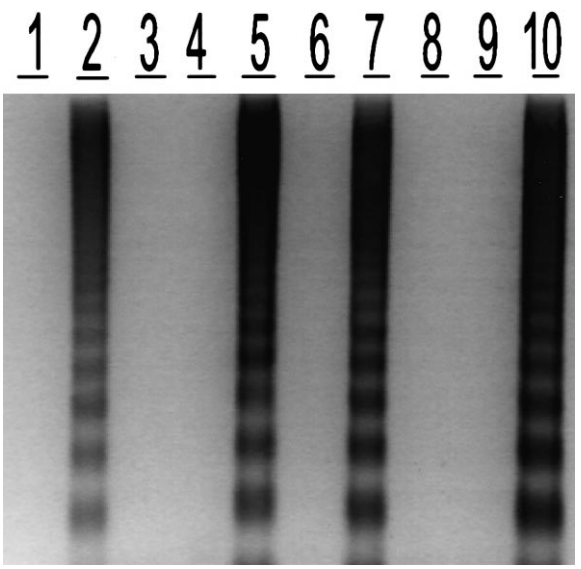


Fig. 3. Analysis by agarose gel electrophoresis of DNA derived from livers (1.8% agarose gel) after staining with ethidium bromide. Lanes 1–5, wt mice; lanes 6–10, caspase-$1^{-/-}$ mice. Lanes 1 and 6, PBS control; lanes 2 and 7, TNF/GalN; lanes 3 and 8, GalN; lanes 4 and 9, mTNF; lanes 5 and 10, anti-Fas.

## 4. Discussion

In order to study the involvement of caspase-1 in apoptosis induced in vivo, we made use of recently generated caspase-$1^{-/-}$ mice [7]. Data about a possible involvement of caspase-1 in TNF- and anti-Fas-induced apoptosis were available [11,12] but the experiments either involved overexpression of caspase-1 or inhibition of apoptosis by caspase inhibitors and not specific caspase-1 inhibitors. For that reason, it could not be concluded whether only caspase-1 plays the lethal role or rather the entire family of caspases.

In this study, we demonstrate that caspase-$1^{-/-}$ mice are equally sensitive to a lethal challenge with TNF+GalN or anti-Fas as the control mice. Furthermore, caspase-$1^{-/-}$ mice underwent apoptosis of hepatocytes and displayed high levels of ALT in the serum to the same extent as control mice. One typical feature of apoptosis, namely the appearance of DNA ladders, also occurred in these caspase-$1^{-/-}$ mice. We conclude from these data that caspase-1 seems not to be involved in the apoptosis induced by TNF+GalN or anti-Fas. These data contradict the results reported earlier. However, in in vitro experiments it was found that all caspases were blocked by the inhibitors [11], whereas in our experiments only caspase-1 is absent. Recently, studies performed with tetrapeptide caspase inhibitors demonstrated that Ac-YVAD-cmk (claimed to be a caspase-1 inhibitor) protected against the lethality and the induction of liver damage evoked by anti-Fas [13]. It was also published that the broad spectrum caspase inhibitor zVAD-fmk confers protection against anti-Fas-induced lethality and liver failure [14] and, finally, that zVAD-fmk prevents anti-Fas- and TNF+GalN-induced lethality and apoptosis [15] in mice. In the different studies reported, the tetrapeptide caspase inhibitors protect against an i.v. challenge with 10 μg anti-Fas, which is comparable to the dose we used. These data contradict our results, but we believe that the inhibitors are 'specific' for a family of caspases

rather than specific for just one caspase. The protection conferred by these inhibitors in the different models must be seen as an inhibition of a whole family, which is completely different from the situation in caspase-1$^{-/-}$ mice, where only this single caspase is absent. Functional redundancy is likely to occur in this situation. In the human system, at least 10 caspases have been identified, all of them differently regulated and with specific substrates. Most certainly, the other caspases play a more prominent role.

We conclude that caspase-1 does not play a crucial role in the induction of apoptosis in hepatocytes of mice by TNF+GalN or anti-Fas. We can further also state that interleukin-18 and interferon-γ do not play an active role in our models tested. The role of caspase-1 might be taken over by the other caspases, or alternatively, caspase-1 might be irrelevant in the induction of apoptosis. Perhaps caspase-1 is important in other models of apoptosis, as it was reported that thymocytes of caspase-1$^{-/-}$ mice completely resist anti-Fas-induced apoptosis [6]. We are currently investigating the possible role of other caspases and are evaluating the use of inhibitors of apoptosis in our models.

*Acknowledgements:* The authors thank Dr. T. Seshadri for providing caspase-1$^{-/-}$ mice. L. Van Geert and E. Spruyt are acknowledged for the animal care and F. Duerinck for purifying mTNF. W.V.M. is a research assistant and P.B. a research associate with the Fonds voor Wetenschappelijk Onderzoek Vlaanderen. Research was supported by the Fonds voor Wetenschappelijk Onderzoek Vlaanderen (Grant G023698N), the Algemene Spaar- en Lijfrentekas and the Interuniversitaire Attractiepolen.

## References

[1] Libert, C., Brouckaert, P. and Fiers, W. (1994) J. Exp. Med. 180, 1571–1575.
[2] Van Molle, W., Libert, C., Fiers, W. and Brouckaert, P. (1997) J. Immunol. 159, 3555–3564.
[3] Ogasawara, J., Watanabe-Fukunaga, R., Adachi, M., Matsuzawa, A., Kasugal, T., Kitamura, Y., Itoh, N., Suda, T. and Nagata, S. (1993) Nature 364, 806–809.
[4] Schulze-Osthoff, K., Ferrari, D., Los, M., Wesselborg, S. and Peter, M.E. (1998) Eur. J. Biochem. 254, 439–459.
[5] Porter, A.G., Ng, P. and Jänicke, R.U. (1997) BioEssays 19, 501–507.
[6] Kuida, K., Lippke, J.A., Ku, G., Harding, M.W., Livingston, D.J., Su, M.S.-S. and Flavell, R.A. (1995) Science 267, 2000–2003.
[7] Li, P., Allen, H., Banerjee, S., Franklin, S., Herzog, L., Johnston, C., McDowell, J., Paskind, M., Rodman, L., Salfeld, J., Towne, E., Tracey, D., Wardwell, S., Wei, F., Wong, W., Kamen, R. and Seshadri, T. (1995) Cell 80, 401–411.
[8] Ghayur, T., Banerjee, S., Hugunin, M., Butler, D., Herzog, L., Carter, A., Quintal, L., Sekut, L., Talanian, R., Paskind, M., Wong, W., Kamen, R., Tracey, D. and Allen, H. (1997) Nature 386, 619–623.
[9] Helle, M., Boeije, L. and Aarden, L.A. (1988) Eur. J. Immunol. 18, 1535–1540.
[10] Leist, M., Gantner, F., Bohlinger, I., Tiegs, G., Germann, P.G. and Wendel, A. (1995) Am. J. Pathol. 146, 1220–1234.
[11] Enari, M., Hug, H. and Nagata, S. (1995) Nature 375, 78–81.
[12] Los, M., Van de Craen, M., Penning, L.C., Schenk, H., Westendorp, M., Baeuerle, P.A., Dröge, W., Krammer, P.H., Fiers, W. and Schulze-Osthoff, K. (1995) Nature 375, 81–83.
[13] Rouquet, N., Pagès, J.-C., Molina, T., Briand, P. and Joulin, V. (1996) Curr. Biol. 6, 1192–1195.
[14] Rodriguez, I., Matsuura, K., Ody, C., Nagata, S. and Vassalli, P. (1996) J. Exp. Med. 184, 2067–2072.
[15] Künstle, G., Leist, M., Uhlig, S., Revesz, L., Feifel, R., MacKenzie, A. and Wendel, A. (1997) Immunol. Lett. 55, 5–10.