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Lipopolysaccharide and D-galactosamine-induced hepatic injury is mediated by TNF-α and not by Fas ligand

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Josephs, Michael D., F. Rena Bahjat, Kunitaro Fukuzuka, Riadh Ksontini, Carmen C. Solorzano, Carl K. Edwards III, Cynthia L. Tannahill, Sally L. D. MacKay, Edward M. Copeland III, and Lyle L. Moldawer. Lipopolysaccharide and D-galactosamine-induced hepatic injury is mediated by TNF-α and not by Fas ligand. *Am J Physiol Regulatory Integrative Comp Physiol* 278: R1196–R1201, 2000.—Tumor necrosis factor (TNF)-α and Fas ligand (FasL) are trimeric proteins that induce apoptosis through similar caspase-dependent pathways. Hepatocytes are particularly sensitive to inflammation-induced programmed cell death, although the contribution of TNF-α and/or FasL to this injury response is still unclear. Here, we report that D-galactosamine and lipopolysaccharide-induced liver injury in C57BL/6 mice is associated with increased hepatic expression of both TNF-α and FasL mRNA. Pretreatment of mice with a TNF-binding protein improved survival, reduced plasma aspartate aminotransferase concentrations, and attenuated the apoptotic liver injury, as determined histologically and by in situ 3′ OH end labeling of fragmented nuclear DNA. In contrast, pretreatment of mice with a murine-soluble Fas fusion protein (Fasfp) had only minimal effect on survival, and apoptotic liver injury was either unaffected or exacerbated depending on the dose of Fasfp employed. Similarly, mice with a spontaneous mutation in FasL (B6Smn.C3H-*Fasl*^{gld} derived from C57BL/6) were equally sensitive to D-galactosamine/lipopolysaccharide-induced shock. We conclude that the shock and apoptotic liver injury after D-galactosamine/lipopolysaccharide treatment are due primarily to TNF-α release, whereas increased FasL expression appears to contribute little to the mortality and hepatic injury.

apoptosis; hepatitis; sepsis; shock

APOPTOSIS (programmed cell death) is a highly conserved process used by multicellular organisms to eliminate those cells that are either unnecessary or potentially injurious to the host (13). Furthermore, apoptotic injury can also occur in acute inflammation. Recent attention has focused on two cytokines, tumor necrosis factor (TNF)-α and Fas ligand (FasL), in inflammation-induced cell killing. The TNF-type I receptor (TNFR-I) and Fas/APO-1/CD95 are both members of the TNF/nerve growth factor-receptor superfamily, which signal apoptosis via a common intracellular suicide cascade (30–32). Apoptosis and hepatocellular injury occur in livers of mice challenged with natural ligands to TNFR-I and Fas (7, 19, 21, 27, 33).

Endogenous production of FasL and TNF-α has been implicated in T cell-mediated hepatic injury. We have observed that treating mice with a soluble Fas immunoadhesin attenuated hepatic injury in concanavalin A-induced hepatitis (17). Similarly, Kondo et al. (16) reported that soluble Fas immunoadhesins were protective in transgenic mice overexpressing hepatitis surface antigens or in mice pretreated with *Corynebacterium parvum* and challenged with lipopolysaccharide (LPS).

D-Galactosamine (D-GalN)/LPS administration has been frequently used as a model of endotoxemic shock (34). In addition to causing shock, D-GalN/LPS induces fulminant hepatocellular injury in mice (2). However, unlike previous models of hepatitis, which are predominantly T cell mediated, D-GalN/LPS is presumed to be principally a macrophage/monocyte-mediated model of shock and liver injury (6). Earlier studies have demonstrated that secreted 17-kD TNF-α and its binding to the TNFR-I are essential for both the lethality and hepatic injury in this model (20).

The current study examined the contribution of TNF-α and FasL to the hepatic injury in a D-GalN/LPS model using both a pharmacological and genetic approach. The results suggested that although both TNF-α and FasL mRNA are increased in livers of mice treated with D-GalN and LPS, only abrogation of TNF-α afforded mice a significant survival advantage and prevented hepatic injury.

MATERIALS AND METHODS

Reagents. Female C57BL/6, 17–19 g, were purchased from Charles River Laboratories (Wilmington, MA) and female B6Smn.C3H-*Fasl*^{gld} were obtained from the Jackson Laboratories (Bar Harbor, ME). Amgen (Thousand Oaks, CA) provided the TNF-binding protein (TNF-bp) and the soluble Fas immunoadhesin (Fasfp). The TNF-bp consists of two extracellular domains of the type I (p55) TNF receptor covalently linked to polyethylene glycol. This construct has been shown to block the pathologic sequelae of both soluble and cell-associated forms of TNF-α (39, 41). Fasfp consists of the extracellular domain of murine Fas (CD95) linked to the Fc and hinge portion of a human IgG (29). TNF-bp was administered at 1 mg/kg body wt, whereas the Fasfp was adminis-

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tered at either 5, 50, 500, 1,000, or 5,000 μg/kg body wt. These quantities of Fasfp span the range of effective doses employed previously by Kondo et al. (16) as well as the doses we showed to be effective in mice with concanavalin A-induced hepatitis (17). Because of a relative shortage in the amount of Fasfp available for these studies, not all analyses could be performed at the highest dose (5,000 μg/kg body wt.). One milligram per kilogram body weight of TNF-bp neutralizes soluble and membrane-associated TNF-α and protects against LPS shock (40, 41). A lethal dose of 8 mg D-GalN (Sigma Chemical, St. Louis, MO) and 100 ng LPS (*Escherichia coli*, serotype 0111:B4; Sigma) (34) was administered intraperitoneally 30 min after TNF-bp, Fasfp, or pyrogen-free physiological saline (vehicle control).

Treatment groups. Animals were studied at three time intervals after D-GalN/LPS administration ($n = 5–16$/group). Some mice were bled after 90 min, and livers were harvested to determine plasma and liver membrane TNF-α bioactivity. Liver RNA was extracted and assayed for Fas, FasL, TNF-α and Cu/Zn superoxide dismutase (SOD) mRNA using RT-PCR. Additional mice were bled after 6 h for plasma aspartate aminotransferase (AST) levels, and livers were prepared for histological examination. A third group of mice received no further intervention, and survival was assessed at 72 h.

Analytical methods. Blood was obtained from the retroorbital plexus at the prescribed time periods, and the plasma fraction was separated by centrifugation. AST levels were determined from neat and serially diluted plasma samples using a commercial kit (Sigma) modified for the small plasma samples obtained from the mice. Liver membranes were isolated and prepared from fresh livers as previously described (41), and concomitantly obtained plasma samples were assayed for TNF-α bioactivity using the WEHI 164 clone 13 cytotoxicity assay (46). Total liver RNA was isolated by guanidine thiocyanate and acid-phenol extraction as previously described (45). One microgram of RNA was reverse transcribed and amplified (50 U MMLV RT, 2.5 U AmpliTaq DNA polymerase, Perkin-Elmer) using specific oligonucleotide primers for murine Fas (5′-CTG GCT GTG AAC ACT GTG TTC GCT GC, 3′-CTG GAC TTT CTG CTC AGC TGT GTC TTG G), FasL (5′-ATC AGC TCT TCC ACC TGC AGA AGC AAC, 3′-AGT TCA ACC TCT TCT CCT CCA TTA GCA CC), TNF-α (5′-GGT GCC TAT GTC TCA GCC TCT, 3′-CAT CGG CTG GCA CCA CTA GTT), and Cu/Zn SOD as an internal control (5′-GTC TGC GTG CTG AAG GGC GAC, 3′-TCT CCT GAG AGT GAG ATC ACA). PCR products were visualized on ethidium bromide-stained 2% agarose gels.

The harvested livers were fixed in 3% buffered Formalin and embedded in paraffin. Five-micrometer sections were affixed to slides, deparaffinized, and stained with hematoxylin and eosin to assess morphological changes. Additional slides were processed for immunostaining of apoptotic nuclei using the Apotag kit (Oncor, Gaithersburg, MD), as described by the manufacturers. Very briefly, digoxigenin-conjugated nucleotides were catalytically added to nucleosome-sized DNA fragments by a terminal deoxynucleotidyltransferase. The 3′ ends of the fragments were then labeled with a fluorescein-conjugated anti-digoxigenin antibody. Nuclei were counterstained with propidium iodide.

RESULTS AND DISCUSSION

Administration of D-GalN and LPS resulted in significant mortality and hepatic injury (Table 1 and Fig. 1). Approximately 30% of the placebo-treated C57BL/6 mice administered D-GalN and LPS survived 72 h. At 6 h, plasma AST levels exceeded 750 Sigma-Frankel U/ml (normal being <50 U/ml) and in situ staining of fragmented DNA revealed patchy areas of apoptotic nuclei (Fig. 2). In the livers of mice treated with D-GalN and LPS, there was also upregulation of both TNF-α and FasL mRNA, although the increased FasL mRNA levels were more modest compared with TNF-α (Fig. 3). In contrast, Fas mRNA was present in livers from healthy animals, and levels were unchanged by D-GalN/LPS treatment. These findings are, therefore, consistent with an endotoxin-induced upregulation of both TNF-α and FasL in the livers of mice. In fact, bioactive TNF-α was recovered from both the plasma and from liver membrane preparations from these animals at 90 min (Fig. 4). Although the current studies do not identify the cell populations expressing either FasL or TNF-α mRNA, they are likely resident macrophages and, possibly, infiltrating inflammatory cells such as natural killer (NK) and T cells. Liles and colleagues (15, 23) recently reported that activated macrophages and blood monocytes express FasL mRNA.

Table 1. *Survival to D-galN and LPS-induced shock: effect of FasL and TNF-α blockade*

	Survival/Total	Statistics
C57BL/6 (LPS/D-GalN)	5/16 (31%)	
+5 μg/kg BW Fasfp	5/10 (50%)	
+50 μg/kg BW Fasfp	7/16 (44%)	$P > 0.05$ vs. LPS/D-GalN
+500 μg/kg BW Fasfp	5/15 (31%)	
+1,000 μg/kg BW Fasfp	6/16 (38%)	
+5,000 μg/kg BW Fasfp	3/6 (50%)	
+1,000 μg/kg BW TNF-bp	10/10 (100%)	$P < 0.001$ vs. LPS/D-GalN
B6Smn.C3H-*Fasl*^{gld} (LPS/D-GalN)	1/10 (10%)	$P > 0.05$ vs. LPS/D-GalN

D-GalN, D-galactosamine; LPS, lipopolysaccharide; FasL, Fas ligand; TNF, tumor necrosis factor; BW, body wt; Fasfp, Fas immunoadhesion.

The question that immediately arises is if the expression of both cytokines is increased in livers from D-GalN/LPS-challenged mice, what are their relative contributions to the mortality and apoptotic injury that occurs. After all, Mignon et al. (24) reported using an identical model that lethality was secondary to a

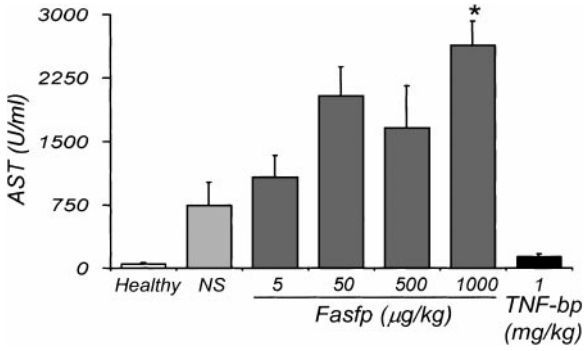

Fig. 1. Six-hour plasma aspartate aminotransferase (AST) levels. AST levels were significantly increased in mice treated with D-galactosamine and lipopolysaccharide, and concentrations were further increased by pretreatment with Fas immunoadhesion [fp; 1,000 μg/kg body wt Fasfp vs. normal saline vehicle control (NS), *$P < 0.05$]. Pretreatment with 1 mg/kg body wt of tumor necrosis factor (TNF)-binding protein (bp) prevented hepatocellular injury.

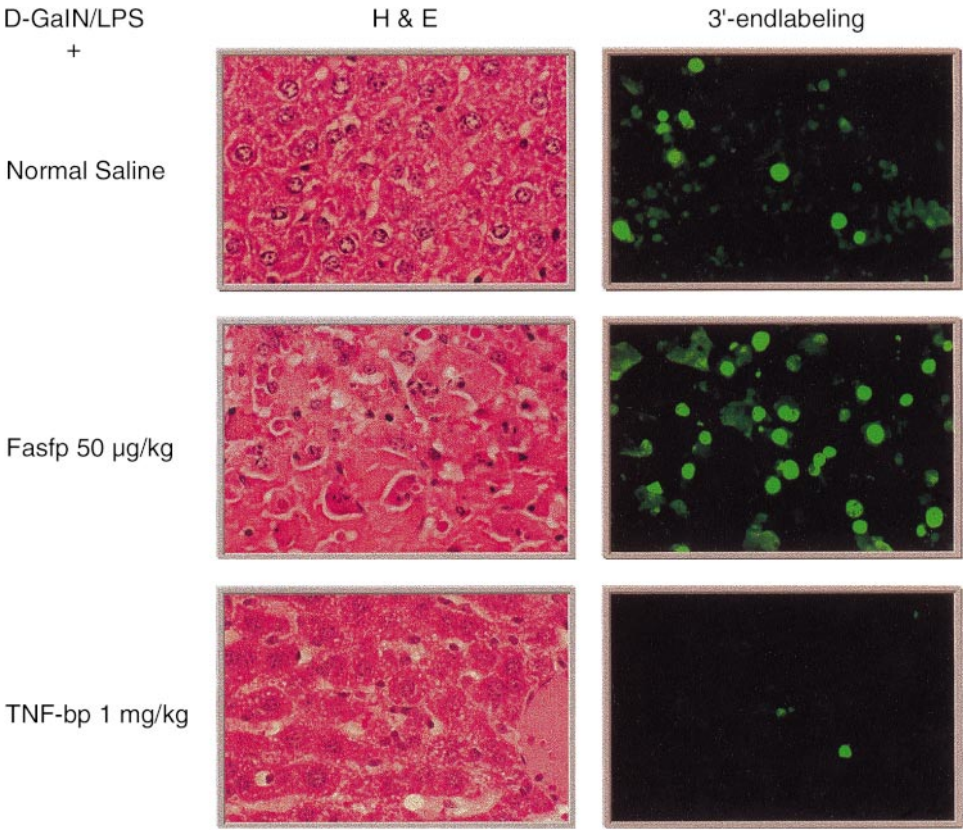

Fig. 2. Photomicrographs (×400) of Formalin-fixed liver sections obtained from mice 6 h after D-galactosamine (D-GalN)/lipopolysaccharide (LPS) challenge. Formalin-fixed and paraffin-embedded sections were stained with hematoxylin (H) and eosin (E) for morphologic examination. Fluorescent 3′-end labeling of nuclear DNA fragments was employed to detect presence of fragmented nuclei representative of apoptotic cells. D-GalN/LPS induced a mild neutrophil infiltrate, and apoptotic nuclei were present throughout liver parenchyma. Pretreatment of mice with Fasfp exacerbated hepatic injury, represented by hemorrhagic necrosis and dilitation of bile canaliculi on hematoxylin and eosin sections and markedly increased numbers of apoptotic nuclei. In contrast, livers from mice receiving TNF-bp preserved normal histological architecture, and apoptosis was nearly abolished.

caspase-3-dependent apoptotic process. The relative contributions of TNF-α and FasL to hepatic injury and outcome in other models is very controversial. In concanavalin A-induced hepatitis, we demonstrated that the contribution of TNF-α to hepatic injury appeared to predominate (17, 41). Those findings confirmed earlier studies with anti-TNF-α monoclonal antibodies and knockout mice lacking either TNF-α or its p55 receptor, which were also protective (8, 9, 18, 21). However, other investigators have failed to show a specific role for TNF-α in this model (43) and have argued that hepatic injury to concanavalin A is primarily FasL dependent (37, 42). In our hands, when mice were challenged with concanavalin A and treated simultaneously with an inhibitor of matrix metalloproteinase, which is presumed to stabilize membrane-associated FasL protein (14), Fasfp protected against the increased injury (17). However, Fasfp was ineffective in mice treated with concanavalin A alone. These latter data are more consistent with the findings of Watanabe et al. (47), who observed that the hepatic injury secondary to concanavalin A was more perforin than FasL dependent.

In the present report, we observed that blocking an endogenous TNF-α response with TNF-bp completely prevented mortality to D-GalN and LPS, a finding that has been reported elsewhere with TNF-α immunoadhesins (22). TNF-α blockade also significantly reduced plasma hepatocellular injury, as reflected by diminished AST concentrations, and decreased number of apoptotic nuclei in the liver, thus confirming an essential role for TNF-α in this model. However, Kondo et al.

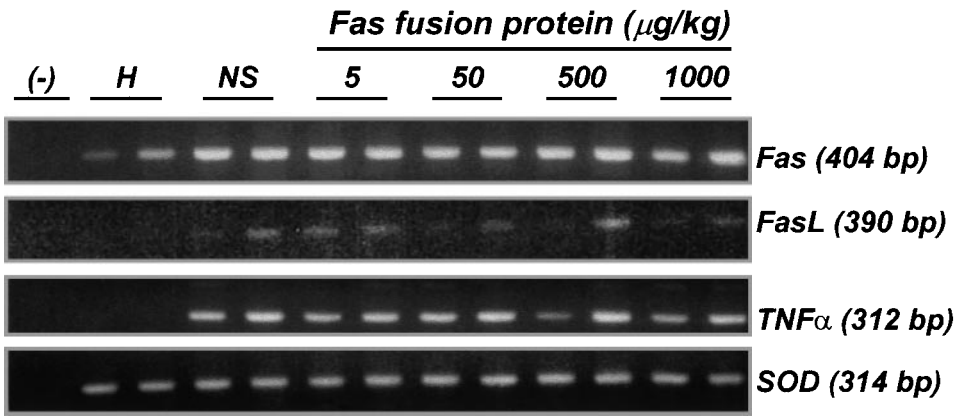

Fig. 3. Ethidium bromide-stained 2% agarose gels demonstrating RT-PCR products from livers of mice. Liver RNA was isolated by guanidine thiocyanate and acid-phenol extraction, and RNA was reverse transcribed and amplified using specific oligonucleotide primers for murine Fas, Fas ligand (FasL), TNF-α, and Cu/Zn superoxide dismutase (SOD; internal control). (−), negative control; H, healthy mice. D-GalN/LPS increased TNF-α, Fas, and FasL mRNA levels, and blocking an endogenous FasL response had no effect on this response.

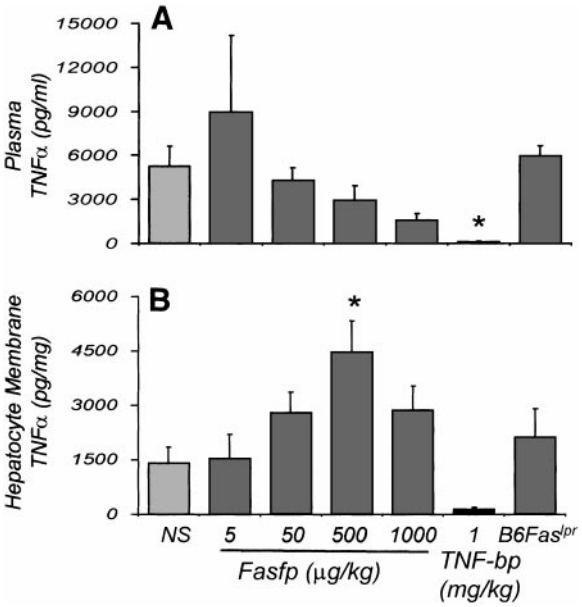

Fig. 4. Plasma and membrane TNF-α levels. Ninety minutes after D-GalN/LPS treatment, bioactive plasma and liver membrane TNF-α levels were determined. Blocking an endogenous FasL response with increasing amounts of Fasfp resulted in a progressive decline in plasma TNF-α and accumulation of TNF-α on liver membranes (*$P <$ 0.05 at 500 μg/kg body wt). In contrast, TNF-bp blocked all TNF-α activity in plasma and liver membranes. *FasL* null mice had plasma and liver membrane levels of TNF-α comparable to those seen in wild-type mice.

(16) recently reported that blocking an endogenous FasL response with similar soluble Fas fusion proteins protected against liver injury in a transgenic mouse model overexpressing hepatitis surface antigens and also prevented mortality and liver injury in a *Corynebacterium parvum* and LPS shock model. In their studies, blockade of FasL was as effective as TNF-α blockade in preventing liver injury and mortality. The authors concluded that FasL-mediated hepatic injury may be a generalized response to inflammation.

Those studies challenge the findings in the present report in which FasL blockade offered no protection. In fact, depending on the dose employed, Fasfp exacerbated hepatic injury after D-GalN/LPS administration, illustrated by increased plasma AST concentrations (Fig. 1), hepatic architectural destruction, and apoptosis (Fig. 2). Similarly, mortality was significant in mice with a mutated form of FasL that presumably prevents Fas signaling (B6Smn.C3H-*Fasl*^{gld}). The discrepancy between the current results and those previously reported by Kondo and colleagues likely reflects the difference in experimental models. Although the authors proposed that the beneficial effect of FasL blockade in the two models (hepatitis B surface antigen overexpression and *Corynebacterium parvum* primed and LPS stimulated) represented a generalized response to inflammation; in actuality, both are T cell-dependent models of hepatic injury. Treatment of mice with *Corynebacterium parvum* results in macrophage activation and increased TNF-α production, but this process is dependent on infiltrating T cells or T cell lymphokines (26) and is more similar conceptually to the concanavalin A-induced model of liver injury. In contrast, the D-GalN/LPS model is predominantly a macrophage-mediated hepatic injury model (5). Morikawa et al. (27) have reported that *lpr* mice lacking functional CD95 were not resistant to D-GalN/LPS-induced lethality and hepatic apoptosis, consistent with the observations reported here. Thus the findings suggest that, depending on the experimental model and the effector cells present, both TNF-α and/or FasL can independently induce hepatic apoptosis.

In mice, TNF-α and FasL both exist as membrane-associated moieties (31). Soluble TNF-α and FasL are generated by proteolysis of the membrane-bound forms by matrix metalloproteinases (MMP) (10, 14, 44). MMP inhibitors reduce mortality from D-GalN/LPS-induced shock in rodents (11), yet hepatitis and hepatic apoptosis are generally unaffected (40, 41). In concanavalin A-induced hepatitis, an MMP inhibitor exacerbated hepatocellular necrosis and apoptosis despite >90% reduction in plasma but only a modest reduction in liver membrane TNF-α concentrations (41). In contrast, TNF-bp, which binds to and blocks the activity of both soluble and cell-associated forms of TNF-α, attenuates both D-GalN/LPS- and concanavalin A-induced hepatitis in the presence and absence of an MMP inhibitor. In concanavalin A-induced hepatitis, Kusters and colleagues (18) demonstrated that a membrane-associated form of TNF-α contributed to the hepatic injury.

It was surprising to observe a substantial elevation in membrane TNF-α concentrations in D-GalN and LPS mice treated with increasing quantities of Fasfp (Fig. 4). There was also a concurrent trend towards reduced plasma TNF-α concentrations, suggesting a possible inhibition in the processing of TNF-α from its membrane to soluble forms. In fact, there was a strong associative relationship between the increased membrane concentration of TNF-α and the degree of liver injury in mice treated with Fasfp (Figs. 1 and 4). An immediate explanation for the observation is not forthcoming, but we postulate that FasL may activate MMPs in a manner analogous to TNF-α (12). FasL has intrinsic proinflammatory properties involving both interleukin (IL)-1 processing and recruitment of neutrophils and macrophages (4, 25, 36). Because upregulation of MMPs is a common observation in inflammation, it is logical to hypothesize that FasL is regulating MMP synthesis or processing. Inhibition of FasL may thus prevent activation of TNF-specific MMPs and enhance membrane-associated TNF-α at the cost of the secreted form. A disintegrin that processes membrane-associated TNF-α to its soluble form has been described (1, 28), but little is known about its regulation.

In summary, hepatocellular injury preceding multiple system organ dysfunction is not an uncommon consequence of endotoxemic shock. The current study supports the contention that although both TNF-α and FasL expression are increased in endotoxemic shock, mortality and the hepatic injury that accompany this model are primarily TNF-α and not FasL dependent. Coupled with earlier published data, the current find-

ings suggest that TNF-α and other T_H1 cytokines, including interferon-γ and IL-12 and -18 (3, 35, 38), appear to play critical roles in the mediation of endotoxin-induced liver injury, presumably through activation of Kupffer and infiltrating NK cells.

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