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Massive liver necrosis after provocation of imbalance between Th1 and Th2 immune reactions in osteopontin transgenic mice

Sumie Mimura[1], Satoshi Mochida[1], Mie Inao[1], Atsushi Matsui[1], Sumiko Nagoshi[1], Takayuki Yoshimoto[2], and Kenji Fujiwara[1]

[1]Gastroenterology and Hepatology, Internal Medicine, Saitama Medical School, 38 Morohongo, Moroyama-cho, Iruma-gun, Saitama 350-0495, Japan
[2]Intractable Disease Research Center, Tokyo Medical University, Tokyo, Japan

Background. Massive liver necrosis can develop as a consequence of imbalance between T-helper (Th)1 and Th2 immune reactions in the liver. Osteopontin is a glycoprotein secreted for the initiation of the Th1 immune reaction, as well as for extracellular matrix formation and calcium deposition in the bone and kidney. Osteopontin is overexpressed in Kupffer cells, macrophages, and stellate cells activated in injured livers. We established transgenic mice expressing osteopontin exclusively in hepatocytes, using a vector containing human serum amyloid P component promoter. The relation of Th1/Th2 immune imbalance to massive liver necrosis was studied using these transgenic mice. ***Methods.*** Transgenic mice and C27BL/6 mice, wild-type controls of the transgenic mice, were given an intravenous injection of concanavalin-A, and the histological extent of liver injuries and plasma cytokine levels were evaluated. ***Results.*** When the transgenic mice received concanavalin-A, massive necrosis and mononuclear cell infiltration developed in the liver, the extent of which was greater in the female mice than in the male mice. This treatment produced minimal liver injury and focal liver necrosis in male and female C57BL/6 mice. In these transgenic and control mice, plasma concentrations of interleukin (IL)-10 and interferon (IFN)-γ were increased after concanavalin-A treatment. However, the upregulation of plasma IL-10 concentration was smaller in the male and female transgenic mice than in the control mice, and the upregulation of the IFN-γ concentration was greater in the female transgenic mice than in the female control mice. ***Conclusions.*** Th1 and Th2 immune reactions were deranged after concanavalin-A treatment, with Th1 immunity predominating in transgenic mice expressing osteopontin in hepatocytes; this immunological imbalance may contribute to massive liver necrosis.

Key words: concanavalin-A, IFN-γ, IL-10, osteopontin, transgenic mouse

Introduction

In Japan, fulminant hepatitis is defined as a critical liver disease, showing hepatic encephalopathy and a prothrombin time of less than 40% of normal values within 8 weeks of the disease onset. The main causes of fulminant hepatitis are viral hepatitis, autoimmune hepatitis, and drug allergy, but the etiology still remains unknown in one-third of the patients.[1] Pathological features of fulminant hepatitis are characterized by massive or submassive necrosis with inflammatory cell infiltration in the liver.[2] Thus, the mechanisms of development of the massive and submassive liver necrosis in fulminant hepatitis must be clarified to establish therapeutic strategies. There are three proposals regarding the regulatory mechanisms of this development; viral factors, immunological factors, and local factors in the liver.

In general, hepatitis virus itself is not cytotoxic to hepatocytes. Liver injury in viral infection occurs during eradication of virus-infected hepatocytes by cytotoxic T lymphocytes (CTLs). CTLs can recognize virus-related antigens on hepatocytes and damage these hepatocytes via the perforin/granzyme B system.[3] This hepatocyte damage is further intensified by CTLs through the Fas/Fas ligand system and tumor necrosis factor (TNF)-α.[3] Similar mechanisms may be involved in the development of autoimmune hepatitis and drug-induced liver injury, because CTLs could recognize auto-antigens and drug-related antigens expressed on hepatocytes.

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Reprint requests to: K. Fujiwara

Fulminant hepatitis is suggested to develop as a consequence of an imbalance between T-helper (Th)1 and Th2 immune reactions, because the expression of interferon (IFN)-γ, a representative Th1 cytokine, was shown to be upregulated in hepatic as well as circulating lymphocytes in patients with fulminant hepatitis.[3,4] Stimulation of the Th1 immune reaction can induce activation of CTLs, Kupffer cells, and hepatic macrophages, while Th1 immunity is attenuated by the Th2 immune reaction.[5] Activated CTLs may aggravate hepatocyte apoptosis,[5,6] and activated Kupffer cells and macrophages can provoke massive liver necrosis through microcirculatory disturbance, due to endothelial cell destruction and fibrin deposition in the hepatic sinusoids.[7,8]

Osteopontin, a secreted glycoprotein that has a binding capacity to hydroxyapatite and calcium, is expressed mainly in the bone and kidney.[9,10] This protein is shown to contribute physiologically to extracellular matrix formation and calcium deposition in these organs.[10] Also, osteopontin can bind to αvβ3 integrin on monocytes and macrophages through the RGD motif,[11] and promote migration of these cells both in vitro and in vivo.[11,12] Moreover, osteopontin is reported to act as a cytokine that is essential for the initiation of the Th1 immune reaction in mice.[13] Previously, we demonstrated that osteopontin expression was upregulated in Kupffer cells, macrophages, and stellate cells activated in injured rat liver,[14,15] suggesting that this factor may be involved in the initiation of the Th1 immune reaction in the liver under pathological conditions. To prove this hypothesis, we established four lines of transgenic mice expressing osteopontin exclusively in hepatocytes, using a vector containing human serum amyloid P component (SAP) promoter, and found that the Th1 immune reaction was activated and CTL infiltration developed spontaneously in the liver.[16]

In the present study, using the transgenic mice, we assessed the relation of the imbalance between Th1 and Th2 immune reactions to massive liver necrosis.

Materials and methods

Osteopontin transgenic mice

Three lines of osteopontin transgenic mice, the offspring of SAP-osteopontin 17, 43, and 73,[16] were maintained on a commercial pelleted diet and water ad libitum in a room at 22°C ± 2°C under normal laboratory lighting conditions. The male and female transgenic mice in the same lines were mated with each other at 8 weeks of age, and genomic DNA extracted from the tails of newborn mice was subjected to polymerase chain reaction (PCR) assay to detect the transgene. Sense and antisense primers were synthesized, based on the cDNA sequences of the 5′ noncoding region of the rabbit β-globin gene and the osteopontin gene, respectively: 5′-TGC TGT CTC ATC ATT TTG GC-3′ for the sense primer and 5′-GCA GGC TGT ATA GCT TCT CCT-3′ for the antisense primer. The mice carrying the transgene at 8 weeks of age were subjected to the experiments. Also, male and female C57BL/6 mice (Japan SLC, Hamamatsu, Japan) at 8 weeks of age were used for the experiments as wild-type controls of the transgenic mice. All animal protocols conformed to the Guide for Care and Use of Laboratory Animals of the National Academy of Sciences.

Experimental design

The mice were given an intravenous injection of 0.25 mg concanavalin-A (Sigma Chemical, St. Louis, MO, USA), dissolved in 0.1 ml saline, through the tail vein. They were scarified 2 h later under anesthesia with ether, and blood was collected through the inferior caval vein with a syringe containing a 1:10 volume of 3.8% sodium citrate, for preparation of plasma to determine TNF-α and interleukin (IL)-10 concentrations. In another experiment, the mice were killed at 12 h after concanavalin-A administration, and blood was collected similarly, followed by excision of the liver. The plasma samples separated from blood and excised livers were used for the measurement of alanine aminotransferase (ALT) and IFN-γ concentrations, and histological examination, respectively. Blood was also collected from mice without concanavalin-A administration for the determination of plasma concentrations of TNF-α, IL-10, and IFN-γ.

Measurement of ALT, TNF-α, IFN-γ, and IL-10 concentrations in plasma

Plasma ALT activity was determined using a commercial kit (Iatron Laboratories, Tokyo, Japan). Plasma concentrations of TNF-α, IFN-γ, and IL-10 were measured using enzyme-linked immunosorbent assay (ELISA) kits (AN'ALYZA Immunoassay System; TECHNE, Minneapolis, MN, USA).

Histological examination of the liver

Excised livers were fixed in 20% formalin neutral buffer solution (Muto Pure Chemicals, Tokyo, Japan) and embedded in paraffin. Sections (4-μm) of each block were deparaffinized, and observed by light microscopy after staining with hematoxylin and eosin. For Morphometry of the extent of liver injury, photographs of the liver specimens were taken under magnification at

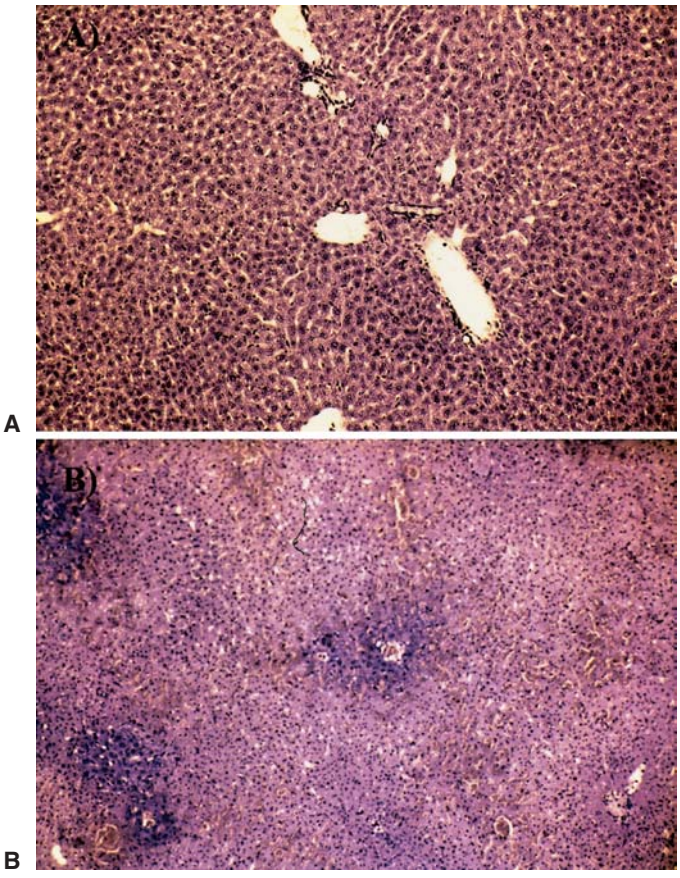

Fig. 1A,B. Histological features of the liver in mice given intravenous concanavalin-A. Mice were given 0.25 mg of concanavalin-A through the tail vein, and scarified 12 h later for histological examination of the liver. **A** Male CL57B/6 mouse. No necrotic lesions are seen in the liver. **B** Female transgenic mouse of the serum amyloid P component (SAP)-osteopontin 17 line. Massive necrosis with mononuclear cell infiltration is present in the liver. ×100

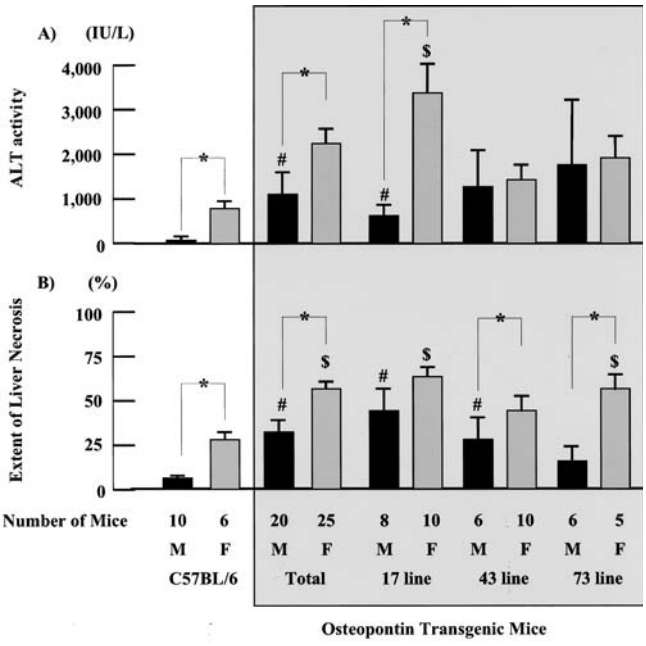

Fig. 2A,B. Plasma alanine aminotransferase (*ALT*) activity and the extent of liver necrosis in mice given intravenous concanavalin-A. Mice were given 0.25 mg of concanavalin-A through the tail vein, and scarified 12 h later for the measurement of plasma ALT activity and histological examination of the liver. **A** Plasma ALT activity. **B** Histological extent of liver necrosis assessed by the ratio of the necrotic area to the entire hepatic lobule area. *Columns and bars* indicate means and standard errors. *M*, male; *F*, female; *17*, *43*, and *73* signify the SAP-osteopontin 17, 43, and 73 lines, respectively. $^{*}P < 0.05$ between male and female mice; $^{\#}P < 0.05$ vs male C57BL/6 mice; and $^{\$}P < 0.05$ vs female C57BL/6 mice, by Mann-Whitney *U*-test

100×. The ratio of the necrotic area to the whole area of hepatic lobules was measured on a scale plate.

Results

Extent of liver injury in the transgenic mice treated with concanavalin-A

Necrotic areas were not found in the liver, and plasma ALT levels ranged between 20 and 30 IU/l in 18 transgenic mice (3 male and 3 female mice in each of the three lines), as well as in 3 male and 3 female C57BL/6 mice without concanavaline-A treatment. As shown in Fig. 1A, intravenous administration of concanavalin-A produced minimal liver injury in male C57BL/6 mice 12 h later; the ratio (percentage) of necrotic area to the whole area of the hepatic lobules was less than 10% in all mice, and plasma ALT activity ranged between 14 and 122 IU/l. In female C57BL/6 mice, however, necrotic areas covering greater than 10% of the hepatic lobules were present. The ratio of the necrotic area to the hepatic lobule area, and the plasma ALT activity were significantly higher in the female mice (mean ± SD, 25.3 ± 11.6% and 926 ± 150 IU/l, respectively) than in the male mice (6.2 ± 3.5% and 33 ± 33 IU/l, respectively; $P < 0.05$ by Mann-Whitney *U*-test; Fig. 2).

In contrast, massive necrosis with mononuclear cell infiltration developed in the liver in the transgenic mice following concanavalin-A treatment, regardless of the mouse line (Fig. 1B). The ratio of the necrotic area to the area of the hepatic lobules was 29.1 ± 33.5% in the male transgenic mice and 54.1 ± 22.2% in the female transgenic mice, being significantly greater in the females than in the males ($P < 0.05$ by Mann-Whitney *U*-test). These ratios in male and female transgenic mice were significantly greater than those in male and female C57BL/6 mice ($P < 0.05$; Fig. 2B). Plasma ALT activity was also increased markedly in these transgenic mice (male, 1129 ± 2387; female, 2262 ± 1881 IU/l); the activ-

ity in the female transgenic mice was significantly higher than the activities in female C57BL/6 mice and in the male transgenic mice ($P < 0.05$; Fig. 2A).

When the extent of liver injury was assessed in the three lines of transgenic mice, the ratio of the necrotic area to the hepatic lobule area was significantly greater in male and female mice of the SAP-osteopontin 17 line, male mice of the 43 line, and female mice of the 73 line than in C57BL6 mice of the same sex ($P < 0.05$ by Mann-Whitney *U*-test; Fig. 2B). Also, plasma ALT activity was significantly higher in male and female mice of the SAP-osteopontin 17 line than in male and female C57BL/6 mice, respectively ($P < 0.05$; Fig. 2A).

Plasma concentrations of cytokines in the transgenic mice treated with concanavalin-A

Plasma concentrations of IL-10, IFN-γ, and TNF-α were not detectable by each ELISA assay in 18 transgenic mice, or in 6 C57BL/6 mice without concanavalin-A treatment. After concanavalin-A administration, plasma concentrations of IL-10, IFN-γ, and TNF-α were increased both in C57BL/6 mice and in the transgenic mice irrespective of the mouse lines, but there was no sex difference in these upregulated levels of the three cytokines, either in C57BL/6 mice or in the transgenic mice, except for TNF-α concentration in the transgenic mice of the 73 line.

The upregulation of plasma IL-10 concentration after concanavalin-A administration was significantly smaller in both male and female transgenic mice (mean ± SD, 156 ± 48 and 138 ± 34 pg/ml, respectively) than in male and female C57BL/6 mice (222 ± 106 and 300 ± 147 pg/ml, respectively; $P < 0.05$ by Students' *t*-test; Fig. 3A), while such upregulation of plasma IFN-γ concentration was greater in the female transgenic mice than in female C57BL/6 mice, prominently in the mice of the SAP-osteopontin 43 line. The plasma IFN-γ concentration (mean ± SD) in the 43 line was 1888 ± 649 pg/ml in the males and 2048 ± 369 pg/ml in the females, values which were significantly higher than the concentrations in C57BL/6 mice of each sex (1219 ± 285 and 1268 ± 265 pg/ml, respectively; $P < 0.05$).

Plasma TNF-α concentration after concanavalin-A administration was equivalent in C57BL/6 mice (mean ± SD, male, 660 ± 255; female, 876 ± 235 pg/ml) and the transgenic mice of the SAP-osteopontin 17 line (male, 563 ± 96; female, 777 ± 573 pg/ml) and the 43 line (male, 607 ± 78; female, 612 ± 20 pg/ml). However, the concentrations in the transgenic mice of the 73 line (male, 373 ± 73; female, 524 ± 102 pg/ml; $P < 0.05$ by Mann-Whitney *U*-test) were lower compared to those in C57BL/6 mice of each sex ($P < 0.05$; Fig. 3B).

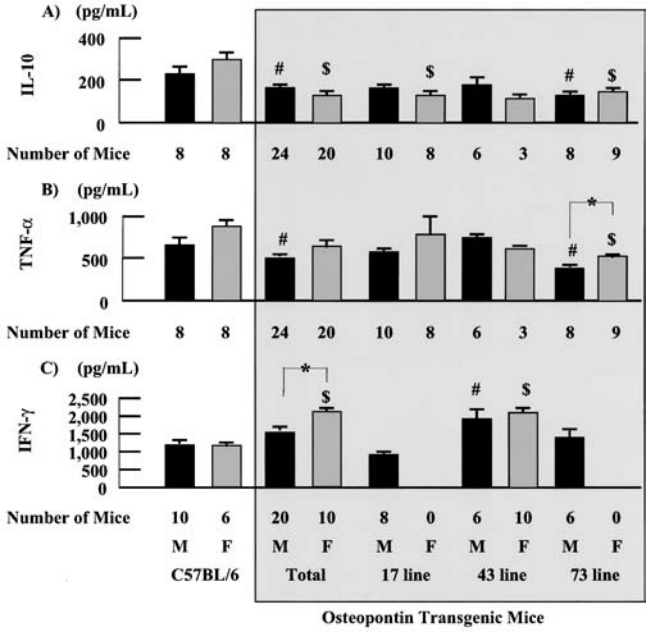

Fig. 3A–C. Plasma interleukin-10 (*IL-10*), tumor necrosis factor-α (*TNF-α*), and interferon-γ (*IFN-γ*) concentrations in mice given intravenous concanavalin-A. Mice were given 0.25 mg of concanavalin-A through the tail vein, and scarified for measurements of plasma IL-10 (**A**) and TNF-α (**B**) concentrations 2 h later, and plasma IFN-γ concentration (**C**) 12 hours later. *Columns and bars* indicate means and standard errors. *M*, male; *F*, female; *17*, *43*, and *73* signify the SAP-osteopontin 17, 43, and 73 lines, respectively. $^{*}P < 0.05$ between male and female mice; $^{\#}P < 0.05$ vs male C57BL/6 mice; and $^{\$}P < 0.05$ vs female C57BL/6 mice, by Students' *t*-test

Discussion

The present study was designed to evaluate the significance of the imbalance between Th1 and Th2 immune reactions in the development of massive liver necrosis, using transgenic mice expressing abundant osteopontin exclusively in hepatocytes, which we established from C57BL/6 mice.[16] Although we had established four lines of transgenic mice, the SAP-osteopontin 17, 43, and 73 lines were used in the present experiments, because breeding efficacy was minimal in the SAP-osteopontin 35 line. In the transgenic mice, osteopontin concentrations in the liver and plasma were about 13 and 2.6 times greater compared to those in the negative littermates, and necrosis with mononuclear cell infiltration, identified as CTLs, developed later than 12 weeks of age,[16] suggesting that the Th1 immune reaction in the liver was activated by the overexpression of osteopontin in hepatocytes. Liver histology was normal and plasma ALT activity was within the normal range (<20 IU/l), regardless of sex, in the transgenic mice until 8 weeks of age,[16] and therefore mice of this age were used for the present experiments.

Th1 and Th2 reactions were stimulated by concanavalin-A, a plant lectin mitogenic for T lymphocytes. Cytokines reflecting Th1 and Th2 immune reactions in the plasma were measured at the time points at which each cytokine showed its maximal concentration, as noted in the related literature. CTL-dependent liver injury has been shown to develop in mice following concanavalin-A administration.[17] IFN-γ and TNF-α, derived from CTLs or Kupffer cells, may be responsible for CTL-dependent liver injury. Also, Kupffer cell depletion and pretreatment with neutralizing antibodies against IFN-γ and TNF-α are reported to attenuate liver injury in mice after concanavalin-A administration.[18–20] In contrast, IL-10 can act as an antagonist of proinflammatory reactions in mice treated with concanavalin-A, because pretreatment with recombinant IL-10 reduced plasma concentrations of IFN-γ and TNF-α, leading to attenuation of liver injury,[21,22] and blockade of IL-10 signaling by a neutralizing antibody aggravated liver injury through the upregulation of plasma IFN-γ and TNF-α concentrations.[23] Thus, plasma concentrations of IFN-γ and TNF-α were measured as Th1 cytokines and the plasma concentration of IL-10 was measured, as a Th2 cytokine, in the present study. Plasma concentrations of IL-10 and INF-α were evaluated 2 h after concanavalin-A administration, and plasma concentration of IFN-γ at 12 h.[24]

The dose of concanavalin-A (0.25 mg/body) adopted in the present experiments was one-sixth of the dose (1.5 mg/body) generally used to provoke massive liver injury in mice.[17] As shown in Fig. 1A and 2, this dose of concanavalin-A produced minimal liver injury in male C57BL/6 mice, but induced focal necrosis with mononuclear cell infiltration in the livers of female C57BL/6 mice. This observation is in line with the report by Takamoto et al.[25] that the extent of concanavalin-A-induced liver injury was more prominent in female mice than in male mice. It is noteworthy, however, that similar treatment with concanavalin-A provoked both massive and sub-massive liver necrosis in the male and female transgenic mice regardless of the lines, and the extent of the necrosis was more prominent in the female mice than in the male mice (Fig. 2). Thus, it is strongly suggested that overexpression of osteopontin in the liver may be an aggravating factor in the liver injury induced by CTLs in mice.

Plasma concentrations of IL-10, IFN-γ, and TNF-α were increased in both male and female C57BL/6 mice following concanavalin-A administration (Fig. 3). Plasma concentrations of these cytokines did not differ between male and female C57BL/6 mice, suggesting that the sex-related difference in concanavalin-A-induced liver injury was not a result of a difference in Th1 and Th2 immune reactions. Also, the plasma concentrations of these cytokines were upregulated in the transgenic mice treated with concanavalin-A. Considering the facts that oophorectomy attenuated concanavalin-A-induced liver injury in female mice, while castration aggravated the injury in male mice,[25] it seems that sex hormones such as estrogen, but not cytokines, may be responsible for such a sex-related difference. On the other hand, the upregulation of plasma IL-10 concentration was less in both the male and female transgenic mice than in the male and female C57BL/6 mice. In contrast, the upregulation of plasma IFN-γ concentration was more prominent in the female transgenic mice than in female C57BL/6 mice. Although the possibility that the overexpression of osteopontin could increase the susceptibility of hepatocytes to Th1 cytokines should be evaluated in future, it is strongly suggested that deranged equilibrium between Th1 and Th2 immune reactions may be a factor that aggravated concanavalin-A-induced liver injury in the transgenic mice.

In the present experiments, the upregulation of plasma TNF-α concentration was smaller in the transgenic mice of the SAP-osteopontin 73 line than in C57BL/6 mice, but it was equivalent in the transgenic mice of the 17 line and the 43 line and the C57BL/6 mice. It is reported that the concentrations of IFN-γ and TNF-α were upregulated in both C57BL/6 and BALB/c mice after concanavalin-A administration,[26,27] and pretreatment with neutral antibody against IFN-γ attenuated plasma ALT activity almost completely in C57BL/6 mice,[26] while anti-TNF-α antibody was more effective than anti-IFN-γ antibody in BALB/c mice.[27] The significance of TNF-α in the development of concanavalin-A-induced liver injury may differ depending on the strains of mice. It seems likely that IFN-γ, but not TNF-α, may play a crucial role in the development of liver injury in the transgenic mice established from C57BL/6 mice.

In conclusion, Th1 and Th2 immune reactions in the liver were deranged, with Th1 immunity predominating, in transgenic mice expressing osteopontin in hepatocytes after concanavalin-A administration, and such an immunological imbalance may contribute to massive liver necrosis.

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