IL-12 induces specific cytotoxicity against regenerating hepatocytes in vivo

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Abstract

Although impaired liver regeneration is thought to be a major cause of death in patients with fulminant hepatitis, the mechanisms are not well defined. Since IL-12 synthesis has been reported to be up-regulated in murine hepatitis virus infection, we studied the influence of continuous IL-12 stimulation on murine liver regeneration using flow cytometric and functional analyses. In non-hepatectomized mice, interestingly, the number of hepatic NK cells was significantly decreased on day 7, after six IL-12 injections, and day 14, after 13 IL-12 injections. The number of hepatic NKT cells was markedly increased on day 7 and day 14 of daily IL-12 treatment. The cytotoxic activity of hepatic lymphocytes against both YAC-1 and p815 cells was enhanced on day 2, after single IL-12 injection, and day 7, after six IL-12 injections. In contrast, hepatic lymphocytes isolated 24 h after partial hepatectomy with IL-12 pretreatment did not show any cytolytic activity against either YAC-1 cells or p815 cells. However, continuous IL-12 stimulation resulted in a significantly higher serum alanine aminotransferase (sALT) level 24 h after the partial hepatectomy as compared with sALT levels in mice subjected to either partial hepatectomy or IL-12 pretreatment alone. On the other hand, the expression of hepatic TNF-α mRNA was markedly enhanced by continuous IL-12 stimulation even 24 h after partial hepatectomy, as compared with that in non-treated mice and hepatectomy alone. Simultaneous administration of anti-tumor necrosis factor (TNF)-α mAb completely inhibited IL-12-induced in vivo enhancement of liver damage after partial hepatectomy. In conclusion, IL-12 induces the specific cytolytic activity against regenerating hepatocytes in vivo mainly through the enhancement of TNF-α synthesis.

Introduction

Impaired liver regeneration has been postulated as one of the major factors responsible for the high mortality among patients with fulminant hepatitis (FH). It has recently been shown that hepatitis B surface antigen (HBsAg)-specific cytotoxic T lymphocytes (CTL) cause a fatal necroinflammatory liver disease in HBsAg-transgenic mice (1,2). This model revealed that antigen non-specific amplification mechanisms play a major role in the spread of liver necrosis (2,3). Although it is speculated that continuous hepatocellular injury may induce impaired liver regeneration, the exact mechanisms responsible for the impaired liver regeneration are not well defined.

Liver regeneration may be regulated cooperatively not only by humoral factors such as hormones, growth factors and growth inhibitory factors, but also by the immune system. It has been reported that regenerating hepatocytes become sensitive to the cytotoxic activity of normal liver-resident NK cells from partially hepatectomized mice (4). It has also been reported that during the acute phase of regeneration after partial hepatectomy in rats, NK cell functions are temporary suppressed, followed by their recovery at the termination of regeneration, suggesting that such selective suppression of NK cell functions during the acute phase represents an important regulatory mechanism for liver regeneration in the presence of hepatic NK cells (5,6). These observations indicate that liver-resident NK cells may be involved in regulating the extent of hepatocyte regeneration.

A recent study revealed that NKT cells proliferate in the
liver after the administration of IL-12 (7), are cytotoxic effector cells and are the main antitumoral lymphocyte population in the liver. NKT cells are thought to be responsible for the recognition and regulation of not only malignant cell proliferation but also benign cell proliferation, suggesting that NKT cells may be involved in the regulation of hepatocyte regeneration (5).

IL-12, characterized as a heterodimeric cytokine produced by antigen-presenting cells, plays a critical role in the regulation of cellular immune responses. *In vitro* (8,9) and *in vivo* (10–12) studies on IL-12 have shown that it enhances the cytotoxic activity of NK cells and CTL. IL-12 also acts as a growth factor for activated NK, T and NKT cells, and promotes the development of Tn1 cells (7,13–15). These observations indicate that IL-12 plays an important role in inflammation. It has also been reported that IL-12 synthesis was up-regulated *in vivo* in murine hepatitis virus (MHV) infection (16), suggesting that IL-12 production is possibly also up-regulated in patients with FH. However, IL-12 does not induce liver damage by itself (17).

We now report that repetitive administration of IL-12 markedly influences the subpopulation of hepatic mononuclear cells (MNC) and exhibits specific cytolytic activity against regenerating hepatocytes in vivo through the up-regulation of tumor necrosis factor (TNF)-α synthesis.

Methods

**Mice**

Male BALB/c (H-2d), B10D2 (H-2d) and C57BL/6 (H-2b) mice (male; age 6–8 weeks; weight range 25–30 g) were obtained from Japan SLC (Shizuoka, Japan).

**Cytokines and antibodies**

Murine recombinant IL-12 was generously provided by Dr Maurice K. Gately (Department of Inflammation/Autoimmune Diseases, Hoffmann-La Roche, Nutley, NJ). FITC-conjugated anti-mouse CD3-ε (hamster IgG), R-phycocerythrin (PE)-conjugated anti-mouse CD122 (IL-2Rβ) chain, rat IgG2b, κ, biotinylated anti-mouse NK1.1 (NKR-P1C) [mouse ([C3H x BALB/c] F1) IgG2a, κ] mAb were purchased from PharMingen (San Diego, CA). Control FITC-conjugated hamster IgG was purchased from Cedarlane (Hornby, Ontario, Canada), and PE-conjugated rat IgG2b, κ was obtained from PharMingen.

**Cell lines**

YAC-1 lymphoma cells and p815 mastcytoma cell line (H-2d) were generously provided by Dr Francis V. Chisari, and were maintained in complete RPMI 1640 medium (Nikken Biomedical, Kyoto, Japan) supplemented with 10% FCS, 5 mM HEPES buffer, 2 mM glutamine, 100 U/ml penicillin, 100 U/ml streptomycin and 0.06 mg/ml anti-PPLO (Gibco BRL, Gaithersburg, MD).

**Treatment of mice with IL-12**

Mice received daily i.p. injections [500 ng, the dose that achieves the increase of hepatic MNC in vivo (17)] of IL-12 in 0.2 ml of PBS (Gibco/BRL) for either 1, 6 or 13 days and were sacrificed 24 h after the final dose was injected. The liver tissues from individual mice were fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections of 5 μm thickness were stained with hematoxylin & eosin for light microscopic evaluation.

**Hepatic and splenic mononuclear cells preparation**

Hepatic and splenic lymphocytes were isolated and purified as previously described (18). Briefly, the excised liver was cut into small pieces with scissors, pressed through a 200-gauge stainless mesh and suspended in complete RPMI 1640 medium. Lymphocytes were separated from parenchymal hepatocytes, hepatocyte nuclei and Kupffer cells by the mouse lymphocyte separation medium, M-SMF (JIMRO, Gunma, Japan), and washed twice in ice cold medium. Splenic cells were obtained by pressing splenic tissue through a 200-gauge stainless mesh and suspended in the medium. The splenic cells were separated from fragmented red blood cells and washed twice in the medium.

**Immunofluorescent staining and flow cytometry**

The MNC were adjusted to a concentration of between 1×10⁶ and 2×10⁶/200 μl in PBS containing 5 mM HEPES buffer. For two-color flow cytometry, cells were incubated with both FITC- and PE-labeled antibodies for 20 min on ice, and washed twice in PBS. For three-color flow cytometry, they were incubated with FITC-, PE- and biotin-conjugated first mAb for 20 min on ice, and washed twice in PBS. They were then incubated with PerCP-conjugated streptavidin for 20 min on ice and washed twice in PBS. The stained cells were analyzed by two- or three-color flow cytometric analysis on a FACScan (Becton Dickinson Immunocytometry Systems, Palo Alto, CA). Data were analyzed using Lysys II software (Becton Dickinson Immunocytometry Systems). We used the expressions of either CD3+ IL-2Rβ+ or CD3- NK1.1+ for identifying NK cells, CD3intermediate (int+) IL-2Rβ+ or CD3int+ NK1.1+ for identifying NKT cells, and CD3high IL-2Rβ- or CD3high NK1.1+ for identifying T lymphocytes. Since BALB/c mice MNC do not express NK1.1 antigen, we analyzed the MNC from BALB/c mice without anti-NK1.1 mAb.

**Cytotoxicity assay**

The cytolytic activities of hepatic and splenic MNC were assessed in a 4 h cytotoxicity assay using europium (Eu)-labeled target cells as previously described (19). YAC-1 cells and p815 cell lines were prepared as targets for the analyses of either NK activity or lymphokine-activated killer (LAK) activity respectively. Target cells were labeled with Eu diethylene-triaminopentaacetate (80 μmol/l; Wako Pure Chemical Industries, Osaka, Japan). Labeled targets (5×10⁵) and various numbers of effector cells were incubated in 96-well round-bottomed plates for 4 h at 37°C. Subsequently, a 20 μl portion of the culture supernatant from each well was mixed with 100 μl of Delfia enhancement solution (Wallac, Turku, Finland) and the released Eu was measured using a time-resolved fluorometer (1230 Arcus; Wallac). The percent Eu release was determined as follows: [(experimental release – spontaneous release)/(total release – spontaneous release)]×100. Spontaneous release was <30% of the maximal release.
RT-PCR analysis
Frozen liver tissue was mechanically pulverized and total hepatic RNA was isolated for the analysis of cytokine mRNA expressions without treatment, 24 h after partial hepatectomy, 24 h after 6 times daily i.p. injection with IL-12 (500 ng/injection/mouse) and 24 h after partial hepatectomy following 6 times daily i.p. injection with IL-12. Total RNA was isolated by the guanidinium isothiocyanate phenol-chloroform method using RNAzol (Tel-Test, Friendswood, TX) was exercised. Then 1 µg of total RNA was reverse transcribed into complementary DNA (cDNA) by using AMV reverse transcriptase (Boehringer Mannheim, Mannheim, Germany). Thermal cycle conditions were 94°C for 45 s for denaturing, 60°C for 45 s for annealing, and 72°C for 2 min for extension. Numbers of amplification cycles were 28 for either TNF-α or G3PDH.

After the last cycle of amplification, the samples were incubated for 7 min at 72°C. The PCR products were electrophoresed on 1.2% agarose gels and stained with ethidium bromide. To ensure that contaminating DNA had not been amplified, PCR without reverse transcriptase was simultaneously demonstrated. Sense and anti-sense primers were based on Clontech Amplimer Set for mouse TNF-α and G3PDH. The quantitation of TNF-α mRNA was performed by competitive PCR using the PCR Mimic Protocol (Clontech, Palo Alto, CA). For semi-quantitative analysis of G3PDH mRNA, dilutions of cDNA were amplified for 20, 23, 25, 28, 30, 32, 34, 36 and 38 cycles to define optimal conditions for linearity and to permit semiquantitative analysis of signal strength.

Role of IL-12 in liver regeneration
A 70% partial hepatectomy was performed in BALB/c mice according to the similar method in rats demonstrated by Higgins and Anderson (20) after 0 or 6 days of IL-12 administration. In order to evaluate the role of TNF-α in the specific cytotoxicity induced by IL-12 against regenerating hepatocytes in vivo, an i.v. injection of TN3 19.12, a mAb specific cytotoxicity induced by IL-12 against regenerating hepatocytes in vivo, an i.v. injection of TN3 19.12, a mAb specific for murine TNF-α (200 µg/mouse) which was generously provided by Dr Robert D. Schreiber (Department of Pathology, Washington University School of Medicine, St Louis, MO) (21), was administered simultaneously with partial hepatectomy. Blood from individual mice was drawn from the tail veins using the cutting method at various time points. Hepatocellular injury was monitored biochemically by measuring serum alanine aminotransferase (sALT) activity.

Statistics
Values were expressed as mean ± SEM. Differences between experimental and control groups were analyzed by the Kruskal–Wallis test followed by Scheffe’s F-test.

Results
Kinetics and lymphocyte phenotypes of hepatic and splenic MNC after repetitive administration of IL-12
Since IL-12 is known to enhance the proliferation of NK, NKT and T cells, we evaluated the kinetics of these cells in vivo after daily IL-12 (500 ng, i.p.) injection into BALB/c mice. The MNC number in both the liver and spleen of BALB/c mice was increased on both day 7 and day 14 after the daily administration of IL-12 (Fig. 1A and B). Although NK cells (CD3– IL-2Rβ+) are the major MNC in non-treated livers, they markedly diminished in number in the liver as well as in the spleen on day 7 and day 14 after the daily administration of IL-12 (Fig. 1A and B). The percentage and number of hepatic NKT cells (CD3int+ IL-2Rβ+) did not change on day 2, whereas they increased dramatically on day 7 and day 14 (Fig. 1A). Splenic NKT cells made a difference in the kinetics, as compared with hepatic NKT cells, since their number was already increased on day 2. The percentage and number of hepatic T cells (CD3high+ IL-2Rβ+) did not change on day 2, increased markedly on day 7 and then decreased to almost within normal range by day 14. On the other hand, the percentage and number of splenic T cells increased on both day 7 and day 14 (Fig. 1A and B). These results, considered together, indicate that the effect of IL-12 depends on the duration of stimulation, the cell type and the organ from which the MNC are isolated. Continuous IL-12 stimulation surprisingly suppressed the proliferation of NK cells and NKT cells replaced NK cells as the major hepatic MNC in vivo (day 2 may be called the ‘NK phase’ and day 7 the ‘NKT/T phase’ in the liver).

Enhancement of the NK activity of MNC by IL-12
To examine whether continuous IL-12 stimulation enhances the cytolytic activity of MNC in vivo, BALB/c mice were administered i.p. injections of IL-12. Either hepatic and splenic macrophages were eliminated by the method for lymphocyte’s isolation. As shown in Fig. 2, hepatic and splenic lymphocytes derived from non-treated mice did not show any cytotoxicity against YAC-1 cells. On day 2, after a single IL-12 injection, MNC from both the liver and spleen exhibited NK activity against YAC-1 cells (Fig. 2). On day 7, after six injections, hepatic MNC exhibited cytolytic activity against YAC-1 cells. In contrast, the splenic MNC did not exhibit any cytotoxicity, although there was no significant difference in the percentage of either splenic NK cells (CD3+ IL-2Rβ+) or NKT cells (CD3int+ IL-2Rβ+) between day 2 and day 7 (data not shown). These results indicate that IL-12 causes proliferation and stimulation of hepatic MNC specifically in the liver, as compared with the effect on splenic NKT and NK cells.
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Fig. 1. The evolution of total number of MNC and cell number of each lymphocyte subset isolated from the liver (A) and spleen (B). The actual cell number per mouse was counted in each subset (NK cell: CD3− IL-2Rβ+; NKT cell: CD3int IL-2Rβ+; and T cell: CD3+ IL-2Rβ−) after daily IL-12 injection into BALB/c mice. Each bar and error bar represent the mean of results in four mice and the SEM respectively. All points marked (*P < 0.05, **P < 0.01) are significantly different from the corresponding group (day 0).

Effect of IL-12 on immunosuppression during the acute phase of liver regeneration

Since a previous study reported that NK cell functions were dramatically inhibited during the acute phase of liver regeneration following partial hepatectomy (6), we also investigated the cytotoxic activity of hepatic MNC that were isolated 24 h after hepatectomy. As shown in Fig. 3(A), the cytotoxic activities of the hepatic MNC against YAC-1 cells (NK activity) and p815 cells (LAK activity) were examined on day 8 after daily injection of IL-12 (6-times injection). To evaluate the effect of partial hepatectomy on these cytotoxic activities, hepatic MNC were prepared 24 h after hepatectomy as effector cells. IL-12 was injected i.p. daily until 1 day before the indicated days and hepatectomy was performed 24 h after the final injection. As illustrated in Fig. 3(B), no cytolitic activity against either YAC-1 cells or p815 cells was observed, indicating that IL-12 stimulation does not annul the immunosuppression of the cytotoxic activities of hepatic MNC resulting from partial hepatectomy.

Effect of IL-12 on regenerating hepatocytes in vivo

Since partial hepatectomy induced mechanical liver damage, the sALT level 24 h after partial hepatectomy was 1272 ± 44.2 IU/l (Fig. 4A and B). It was previously (17) reported that IL-12 treatment did not induce a significant increase in sALT activity. To evaluate the effect of continuous stimulation with IL-12 on regenerating hepatocytes, a 70% hepatectomy was performed on day 7 after the daily administration of IL-12. Pretreatment with IL-12 significantly enhanced liver cell injury as assessed 24 h after the hepatectomy, on day 8 after daily injection of IL-12 [sALT activity = 2847 ± 370 IU/l (p < 0.01); Fig. 4B]. This enhancement of hepatocellular injury in IL-12-pretreated mice rapidly began to decrease 48 h after the partial hepatectomy (Fig. 4A). These results indicate that regenerating hepatocytes are more susceptible to IL-12 pretreatment than resting hepatocytes, and that such susceptibility is observed during the acute phase of liver regeneration. Histopathological examination of hematoxylin & eosin-stained tissue sections was also performed 24 h after partial hepatectomy. No histopathological change was observed in mice tissues following hepatectomy alone (Fig. 5A). In contrast, after pretreatment with IL-12 (6-times injection) and partial hepatectomy (on day 7), some hepatocytes exhibited features of apoptosis, with marked MNC infiltration in the hepatic sinusoids and numerous necroinflammatory foci (Fig. 5C). Repetitive IL-12 injection without hepatectomy resulted in few inflammatory foci, although sinusoidal MNC were increased (Fig. 5B). These histological features are thought to correlate with the significant elevation of sALT levels, suggesting that continuous IL-12 stimulation promoted the development of
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Fig. 2. NK activities of hepatic and splenic MNC against Eu-labeled YAC-1 cells. IL-12 (500 ng/mouse) was injected i.p. daily into BALB/c mice until 1 day before the indicated days. The effector cells (hepatic or splenic MNC) were isolated on the indicated days. The NK activity was assessed by a 4-h Eu release assay at the indicated E:T ratios. Each data point and error bar represents the mean of triplicate analysis of pooled MNC obtained from three mice.

Fig. 3. Influence of IL-12 on NK activities of hepatic MNC isolated 24 h after hepatectomy. IL-12 (500 ng/mouse) was injected, i.p. daily into BALB/c mice 6 times until 48 h before the isolation of hepatic MNC. A sham operation was performed 24 h after the final IL-12 injection. The MNC were incubated with Eu-labeled, either YAC-1 cells or p815 cells, for 4 h at an E:T ratio of 40. Each data point and error bar represents the mean of triplicate analysis of pooled MNC obtained from three mice (A). IL-12 (500 ng/mouse) was injected i.p. daily into BALB/c mice 6 times until 48 h before the isolation of hepatic MNC. Partial hepatectomy was performed 24 h after the final IL-12 injection. Hepatic MNC were isolated 24 h after the operation. The hepatic MNC were incubated with Eu-labeled YAC-1 cells or p815 cells for 4 h at an E:T ratio of 40. Each data point and error bar represents the mean of triplicate analysis of pooled MNC obtained from three mice (B).
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Fig. 4. The effect of IL-12 treatment on liver regeneration in vivo. sALT levels were monitored on indicated days after partial hepatectomy (day 0) either with or without IL-12 pretreatment (from day –6 to day –1) (A). The point marked (**P < 0.01) is significantly higher than a group of mice not subjected to IL-12 treatment. No significant differences in sALT levels are observed on day 0, day 2 and day 5 after hepatectomy between IL-12-treated and non-treated groups (A). In (B), partial hepatectomy was performed on day 7 of the daily injection with IL-12 and sALT levels were measured 24 h after hepatectomy (on day 8). sALT levels in the groups subjected to IL-12 pretreatment are significantly higher (**P < 0.01) than those in groups subjected to either hepatectomy alone or IL-12 treatment with sham operation. The simultaneous administration of anti-TNF-α mAb with partial hepatectomy into BALB/c mice on day 7 of the daily injection with IL-12 resulted in significant reduction (*P < 0.05) of sALT level 24 h after hepatectomy, as compared with the level in a corresponding group without anti-TNF-α mAb. Each bar and error bar represent the mean of results in four mice and the SEM respectively.

necroinflammatory foci in the liver, by enhancing sinusoidal MNC proliferation.

Role of TNF-α in liver regeneration

As shown in Fig. 6(A), the hepatic mRNA expression of TNF-α was not detectable in both non-treated mice and mice 24 h after partial hepatectomy alone. In contrast, the hepatic mRNA expression of TNF-α was markedly up-regulated by repetitive IL-12 injections in mice treated either with or without partial hepatectomy. Competitive RT-PCR revealed that partial hepatectomy did not suppress hepatic mRNA expression of TNF-α enhanced by repetitive IL-12 administrations as compared with that in mice with IL-12 administration alone (Fig. 6B).

As shown in Fig. 4(B), the simultaneous administration of anti-TNF-α mAb with partial hepatectomy resulted in virtually complete inhibition of the enhancement of liver cell injury on day 8 after the daily administration of IL-12 for 6 days. Also on histopathological analysis, the liver tissues treated with anti-TNF-α mAb displayed neither features of apoptosis nor necroinflammatory foci (Fig. 5D) although sinusoidal MNC infiltration was present.

Discussion

It has recently been shown that HBsAg-specific CTL cause a fatal liver disease in hepatitis B virus transgenic mice (1,2). Based upon the similarities between this model and human FH, we attempted to elucidate the mechanisms of impaired liver cell regeneration, which is thought to be a major cause of death, but the mechanisms remain to be clarified in detail. The retention of hepatitis B virus antigens in the hepatocytes did not by itself affect liver regeneration after partial hepatectomy (unpublished observation). Subacute-type FH shows relatively slow progression of disease as compared with acute-type FH. Duration from the onset of disease to the appearance of hepatic encephalopathy is >10 days in subacute-type FH. In subacute-type FH, hepatocellular necrosis is less severe but the mortality is higher as compared with the acute type. This has been attributed mainly to the lack of sufficient liver cell regeneration in subacute-type FH. Based on these observations, we focused on the role of intrahepatic MNC in liver regeneration in mice.

In the present study, we demonstrated that continuous IL-12 stimulation markedly influenced the number and phenotype of lymphocytes in the liver and spleen (Fig. 1). As shown in Fig. 1, the number of both hepatic and splenic NK cells (CD3– IL-2Rβ+) significantly decreased on day 7 and day 14 of daily IL-12 administration, although NK cells were the major MNC in non-treated liver. On the other hand, the number of hepatic NKT cells (CD3hi IL-2Rβ+) did not change on day 2 of IL-12 administration, whereas it dramatically increased on day 7 and day 14. However, splenic NKT cells increased as early as on day 2. Thus, the influence depended on the duration of IL-12 stimulation, the cell type and the organ from which the lymphocytes are derived. The cytotoxic activity of MNC in both the liver and spleen against YAC-1 cells increased on day 2 of IL-12 injection, although the percentage (data not shown) and number of NK cells was reduced in the spleen and not changed in the liver (Fig. 1). As shown in Fig. 1, the major MNC subset in non-treated BALB/c mice consisted of NK cells in the liver and T lymphocytes (CD3+ IL-2Rβ+) in the spleen. However, in the late phase of continuous IL-12
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Fig. 5. Histopathological evaluation of the liver of IL-12-treated mice 24 h after partial hepatectomy. Histopathological characteristics of the liver on day 7 of the daily i.p. injection with IL-12 without partial hepatectomy (B), 24 h after partial hepatectomy in otherwise non-treated BALB/c mice (A), mice pretreated with daily i.p. injection of IL-12 (6 times injection; C), and mice pretreated with both daily i.p. injections of both IL-12 and anti-TNF-α mAb (D). No histopathological change is observed in (A). Note the marked increase in sinusoidal MNC in (B)–(D) as compared with (A). Necroinflammatory foci are observed in (C, arrowhead), and some hepatocytes exhibit nuclear and cytoplasmic condensation (apoptotic hepatocytes). In contrast, neither necroinflammatory foci nor apoptotic hepatocytes are observed in (B) and (D). All panels stained with hematoxylin & eosin; original magnification ×200. A representative figure derived from the analysis of four mice is shown for each group.

stimulation (on day 7 and day 14), NKT cells replaced NK cells in the liver and the number of T lymphocytes was markedly increased in the spleen. Since the hepatic MNC still exhibited cytolytic activity against YAC-1 cells on day 7, NKT cells were supposed to assume the NK activity in the late phase (on day 7 and day 14; Fig. 2). These results indicate that different subsets of MNC probably affect the inflammation at the respective time points.

As previously described, IL-12 is regarded as the cytokine that initiates cell-mediated immune responses (22) and its synthesis is therefore considered to precede IFN-γ production (22). IFN-γ, on the other hand, has been shown to stimulate IL-12 synthesis in cultured macrophages (23,24), suggesting a positive feedback. Since we reported that IFN-γ production is essential for CTL-induced necroinflammatory liver disease in HBsAg transgenic mice (2) and IL-12 synthesis was actually up-regulated in hepatitis during a MHV infection (16), IL-12 is presumed to play an important role in FH. However, no beneficial effect of IL-12 was demonstrated in mice infected with influenza virus, encephalomyocarditis virus, respiratory syncytial virus (25), lymphocyte choriomeningitis virus (26) or murine cytomegalovirus (27). Furthermore, in vivo anti-viral protection against MHV mediated by IL-12 was much weaker than that by IFN-γ (16). On the other hand, IL-12 administration did not induce progressive hepatocellular necrosis (17). Contrary to expectations, these results suggest that IL-12 does not seem to have an important role in either viral clearance or liver cell injury.

It has been reported that regenerating hepatocytes become sensitive to the cytotoxic activity of normal liver-resident NK cells in partially hepatotomized mice (4). Furthermore, a previous study reported that NK cell functions were dramatically inhibited in the acute phase of liver regeneration following partial hepatectomy (6). NK activity was functionally suppressed with respect to spontaneous killing of YAC-1 cells (NK activity), antibody-coated p815 cells (antibody-dependent cell-mediated cytotoxicity) and regenerating hepatocytes, suggesting that such selective suppression of
NK cell function during the acute phase probably represents an important regulatory mechanism that allows liver regeneration even in the presence of hepatic NK cells. In non-hepatectomized mice, we demonstrated that hepatic MNC maintained NK activity during continuous stimulation with IL-12 (Fig. 2). As shown in Fig. 4, pretreatment of IL-12 resulted in a significantly higher sALT elevation 24 h after the daily administration of IL-12 as compared with that in mice treated with IL-12 alone (Fig. 6B). In addition, the IL-12-induced enhancement of specific cytotoxicity on day 8 was almost completely inhibited by prior administration with anti-mouse TNF-α mAb in vivo (Fig. 4B). These results indicate that TNF-α plays a major role in the specific cytotoxicity against regenerating hepatocytes. TNF-α has been reported to be produced by activated cell populations such as NK, T cells and macrophages. As shown in Fig. 3(A), hepatic NK cells markedly diminished in number on day 7 after the daily administration of IL-12. Thus, the major source of TNF-α may not be NK cells in this model. The exact source of TNF-α is still unknown, but efforts are being made in our laboratory to investigate which cell populations produce TNF-α.

In conclusion, the number of NK cells increases in the early phase and that of NKT cells increases in the late phase of continuous IL-12 administration. However, IL-12 stimulation does not annul the liver regeneration-related suppression of direct cytolytic activity of hepatic lymphocytes. Since IL-12 stimulation actually enhances hepatocellular injury in vivo during early phase of liver regeneration, continuous IL-12 stimulation plays an important role in impaired liver regeneration. The specific cytotoxicity against regenerating hepatocytes in vivo may not be caused by the direct effect of IL-12-stimulated hepatic NK, NKT and T lymphocytes. It is suggested that TNF-α mediated by continuous IL-12 stimulation may play a critical role on the in vivo specific cytotoxicity against regenerating hepatocytes in this model.

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**Abbreviations**

- CTL: cytotoxic T lymphocyte
- FH: fulminant hepatitis
- HBsAg: hepatitis B surface antigen
- LAK: lymphokine-activated killer
- MHV: murine hepatitis virus

- **IL-12 regulates liver regeneration in vivo**
MNC  mononuclear cell  
PE  phycoerythrin  
sALT  serum alanine aminotransferase  
TNF  tumor necrosis factor  

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