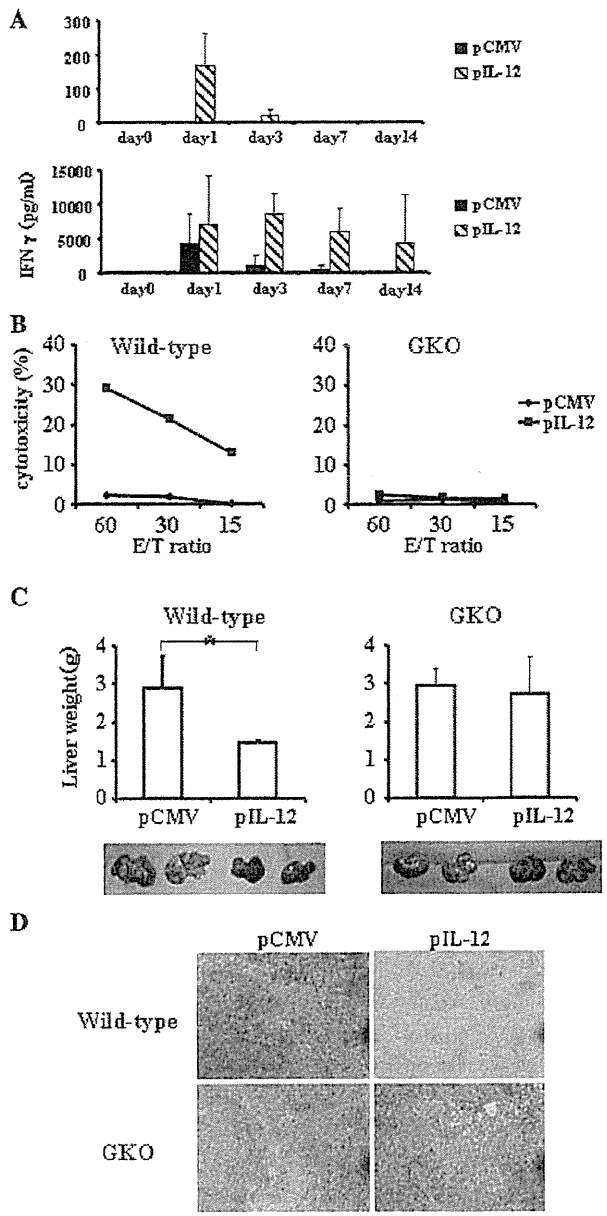

Fig. 1 Effects of hydrodynamic injection of IL-12-encoding plasmid. **a** Wild-type mice were hydrodynamically injected with either pCMV-IL-12 (*hatched bars*) or pCMV (*closed bars*) and bled at the indicated time points to measure the levels of serum IL-12 and IFNγ. Results are indicated as mean and SD ($n = 6$/group). **b** NK-cell activation after IL-12 administration. Hepatic mononuclear cells were isolated from wild-type mice (*left*) or GKO mice (*right*) which had been injected with pCMV-IL-12 (*closed squares*) or pCMV (*closed diamonds*) 4 days earlier. Yac1 lytic ability was measured by a standard ^{51}Cr-release assay at the indicated effector and target ratios (E/T ratio). All experiments were performed at least 3 times and representative data are shown. **c** and **d** Anti-metastatic effects of IL-12 therapy. Wild-type mice (*left*) or GKO mice (*right*) were intrasplenically injected with CT-26 cells and, 2 days later, hydrodynamically injected with either pCMV-IL-12 or pCMV. At 14 days after the plasmid injection, the mice were killed to examine liver tumor development. **c** Data are indicated as mean and SD of the liver weight at the top ($n = 6$/group) and a representative picture of the liver in each group is shown at the bottom. *$p < 0.001$. **d** Representative histology of liver sections

with those from naive mice or pCMV-injected mice. The levels of IFNγ production were highest in NK cells among those cells. Even at a later time point, 7 days after plasmid injection, NK cells were found to produce the highest levels of IFNγ (data not shown).

IL-12-induced STAT4 signaling and IFNγ production increased in NK cells

IL-12 activates Janus kinases Tyk2 and Jak2, STAT4 as well as other STATs. To examine the activation of STAT1 and STAT4, we isolated splenocytes from wild-type mice and GKO mice and stimulated them with IL-12 and/or IFNγ in the presence or absence of anti-IFNγ Ab (Fig. 3a). IL-12 led to phosphorylation of both STAT1 and STAT4 in wild-type splenocytes. In contrast, the same treatment led to phosphorylation of STAT4, but not of STAT1, in GKO splenocytes. Addition of IFNγ restored STAT1 phosphorylation in GKO splenocytes. Furthermore, adding anti-IFNγ inhibited STAT1 phosphorylation in wild-type cells. These findings demonstrated that phosphorylation of STAT4 is a direct effect of IL-12 but phosphorylation of STAT1 is indirect, via an autocrine or paracrine IFNγ-dependent manner.

To examine STAT1 and STAT4 activation and IFNγ production in NK cells and non-NK cells, we prepared whole mononuclear cells as well as NK and non-NK populations from wild-type spleens and stimulated the cells with IL-12 (Fig. 3b). NK cells expressed higher levels of STAT4 than non-NK cells. Upon IL-12 treatment, STAT4 was rapidly phosphorylated in NK cells, but to a lesser extent in non-NK cells. In contrast, NK cells expressed lesser levels of STAT1 than non-NK cells. STAT1 was similarly phosphorylated in NK cells and non-NK cells upon IL-12 treatment. Both NK cells and non-NK cells produced significant levels of IFNγ, but the levels were much higher in NK cells than non-NK cells (Fig. 3c). These results indicated that compared with non-NK cells, NK cells possessed higher levels of STAT4, a direct signaling molecule of IL-12, and produced higher levels of IFNγ than non-NK cells.

NK cells were sufficient for IL-12-mediated anti-tumor effects

The above observation indicated that NK cells are a predominant producer of IFNγ, which was critical for the IL-12-induced anti-tumor effects. To examine whether NK

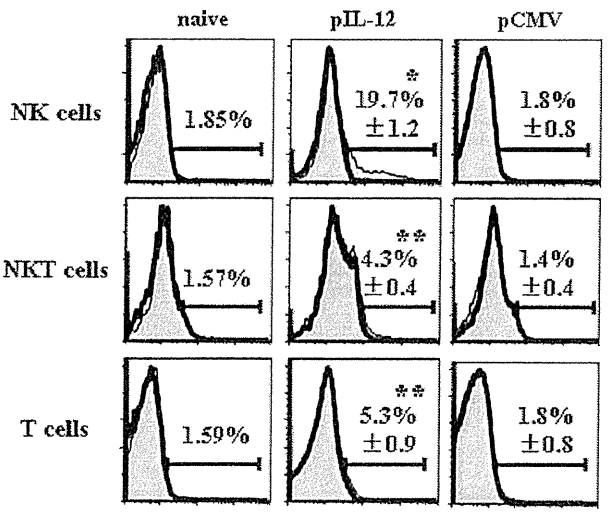

Fig. 2 IFNγ expression of mononuclear cells after IL-12 administration. Wild-type mice were injected with pCMV-IL-12 or pCMV, or were untreated (naive). Mononuclear cells were isolated from the liver 2 days after plasmid injection and stained with anti-TCRβ mAb, anti-DX5 mAb and anti-IFNγ mAb. *Closed histograms* show the IFNγ expression in the gated populations ($TCR\beta^+/DX5^+$ cells for NK cells, $TCR\beta^+/DX5^+$ cells for NKT cells and $TCR\beta^+/DX5^-$ cells for T cells). Isotype control stainings are shown by *open histograms*. Numbers in histograms represent averages ± SD of percentages of positive cells ($n = 3$ mice/group). $^*p < 0.0001$ vs. mock in NK populations. $^{**}p < 0.05$ vs. mock in each population

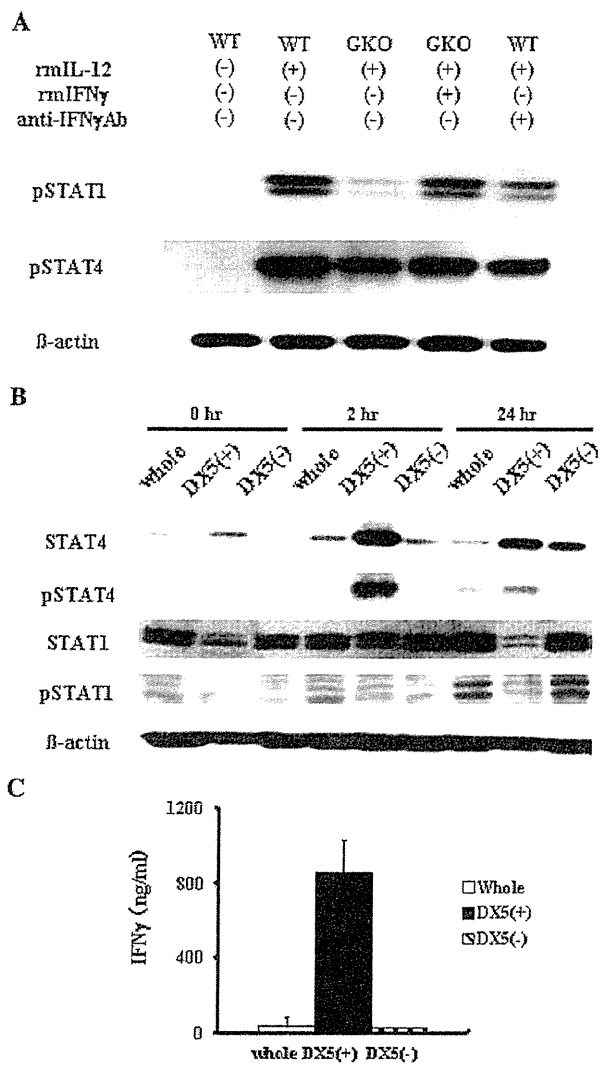

Fig. 3 STAT signaling and IFNγ production of mononuclear cells in vitro treated with IL-12. **a** STAT1 and STAT4 activation of splenocytes in vitro treated with IL-12. Splenocytes were isolated from wild-type mice or GKO mice and treated with or without recombinant IL-12 (20 ng/mL) in the presence or absence of recombinant IFNγ (500 ng/mL) or anti- IFNγ antibody (20 μg/mL) for 24 h. Cellular lysates were analyzed by Western blot for the expression of phospho-STAT1, phospho-STAT4 and β-actin. **b** and **c** STATs expression and signaling of NK cells and non-NK cells. Splenocytes were isolated from wild-type mice. Whole splenocytes were further purified into $DX5^+$ cells and $DX5^-$ cells. Each cell population was cultured with recombinant IL-12 (20 ng/mL) for the indicated times. **b** The cells were lysed to examine expression of whole STAT and phospho-STAT by Western blot. **c** The levels of IFNγ in the culture supernatant at 24 h were determined by ELISA. Data are expressed as mean and SD ($n = 3$)

cells are sufficient for the anti-metastatic effects of IL-12, we examined the anti-metastatic effect in Rag2 KO mice which lack T cells, B cells and NKT cells. pCMV-IL-12 injection enhanced the Yac1 lytic ability of hepatic mononuclear cells in Rag2 KO mice higher than in wild-type mice (Fig. 4a). To examine whether NK cells are sufficient for IL-12-mediated rejection of hepatic metastasis, we injected pCMV-IL-12 or pCMV into mice that had been intra-splenically injected with CT-26 cells 2 days earlier. Serum IFNγ levels of Rag2 KO mice were about 4 times higher than those of wild-type mice (Fig. 4b). pCMV-IL-12 completely suppressed hepatic metastasis in Rag2 KO mice (Fig. 4c).

Adoptive transfer of wild-type NK cells into GKO mice restored the anti-tumor effects of IL-12

Since NK cells were sufficient for producing IL-12-induced anti-tumor effects, we postulated that their production of IFNγ may play an important role in these effects. To test this, we performed adoptive transfer experiments with GKO mice. First, whole mononuclear cells isolated from the spleens of wild-type mice (2.0×10^8 cells) were adoptively transferred to GKO mice 1 day before plasmid injection. pCMV-IL-12 injection increased Yac1 lytic activity of hepatic mononuclear cells in the adoptively transferred group, but not in the untreated group (Fig. 5a). pCMV-IL-12 induced significant increase in serum IFNγ levels 4 days after plasmid injection in the adoptive transferred group, but not in the other groups (Fig. 5b). The

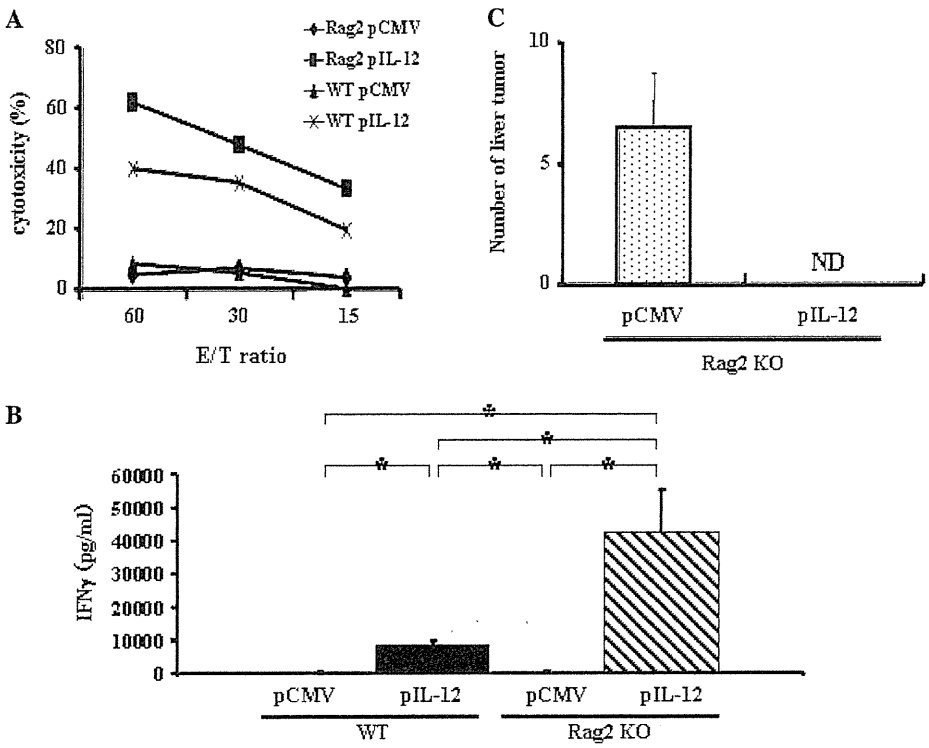

Fig. 4 Anti-tumor effects of IL-12 in Rag2 KO mice. Serum IFNγ levels and NK-cell activation. Wild-type or Rag2 KO mice were hydrodynamically injected with either pCMV-IL-12 or pCMV and killed at 4 days. **a** Yac1 lytic ability of hepatic mononuclear cells was determined by Cr releasing assay as the indicated effector and target ratios (E/T ratio). Experiments were done 2 times and representative data are shown. **b** The levels of serum IFNγ were determined by ELISA. Data are expressed as mean and SD ($n = 7$/group). $^*p < 0.0001$. **c** Anti-metastatic effect. Rag2 KO mice were intrasplenically injected with CT-26 cells and, 2 days later, hydrodynamically injected with either pCMV-IL-12 or pCMV. Fourteen days after plasmid injection, mice were killed to examine tumor development in the liver. The numbers of hepatic tumors in each group are expressed as mean and SD ($n = 7$/group). *ND* not detectable

anti-metastatic effect of IL-12 was restored in GKO mice when whole mononuclear cells from wild-type mice were adoptively transferred (Fig. 5c).

To evaluate the contribution of IFNγ production from each subset of mononuclear cells to the anti-metastatic effect of IL-12, we adoptively transferred the same number of whole mononuclear cells, NK cells or non-NK cells from wild-type mice (4.0×10^6 cells) 1 day before pCMV-IL-12 injection and analyzed liver tumor formation. Only in the NK-cell-transferred group, pCMV-IL-12 injection induced NK cytolytic ability in the liver and IFNγ elevation in serum 4 days after plasmid injection, but not in the other groups (Fig. 5d, e). No liver tumor formed in the NK-cell-transferred group. In contrast, livers in other groups had massive tumors, and the liver weights were significantly heavier than those in the NK-cell-transferred group (Fig. 5f). These results clearly demonstrated the strong impact of IFNγ produced from NK cells on IL-12-induced anti-tumor effects compared with that from non-NK cells.

Anti-tumor effects of IL-12 deteriorated slightly in mice depleted of NK cells

To examine the involvement of NK cells in the tumor deletion by IL-12 therapy, we induced depletion of NK cells by repeatedly injecting anti-asialoGM1 antibody. The cytolytic ability of NK cells was completely abolished in the anti-asialoGM1 antibody-injected group (Fig. 6a). Serum IFNγ induction by IL-12 in the NK depletion group was about half of that in the control immunoglobulin injected group (Fig. 6b). Unexpectedly, pCMV-IL-12 injection inhibited macroscopic liver metastasis of CT-26 cells in NK cell-depleted mice (Fig. 6c). However, a number of microscopic tumor regions were observed after IL-12 therapy in NK cell-depleted mice but not in control IgG-injected mice (Fig. 6d). This finding indicated that NK cells are required for a full-blown IL-12 anti-tumor effect, but IL-12's anti-tumor effect was still observed even if the NK cells were knocked down. To examine the underlying mechanisms of anti-tumor effect in NK cell-depleted mice,

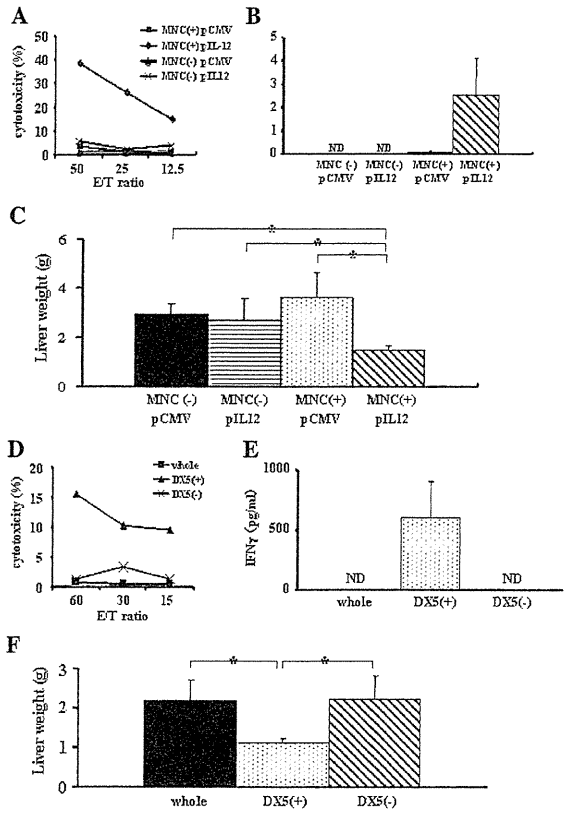

Fig. 5 Adoptive transfer of wild-type cells into GKO mice. Adoptive transfer of wild-type splenocytes restored anti-tumor effects of IL-12 in GKO mice. **a** GKO mice were intravenously injected with or without 2.0×10^8 splenocytes from wild-type mice and, 1 day later, hydrodynamically injected with either pCMV-IL-12 or pCMV. Mice were killed 4 days after plasmid injection. Yac1 lytic ability of hepatic mononuclear cells was expressed as the indicated effector and target ratios (E/T ratio). Experiments were done 3 times and representative data are shown. **b** and **c** GKO mice were intrasplenically injected with CT-26 cells and, 1 day later, intravenously injected with or without 2.0×10^8 splenocytes from wild-type mice. Two days after CT-26 injection, mice were hydrodynamically injected with either pCMV-IL-12 or pCMV. **b** The levels of serum IFNγ 4 days after plasmid injection are expressed as mean and SD (n = 6/group). **c** Fourteen days after plasmid injection, mice were killed to examine liver tumor development by measuring liver weight. The results are indicated as mean and SD (n = 6/group). *ND* not detectable. *$p < 0.01$. Adoptive transfer of wild-type NK cells, but not non-NK cells, restored anti-tumor effects of IL-12 in GKO mice. **d** Wild-type splenocytes were purified into $DX5^+$ cells and $DX5^-$ cells. GKO mice were intravenously injected with 4.0×10^6 whole mononuclear cells or $DX5^+$ cells or $DX5^-$ cells and, 1 day later, hydrodynamically injected with either pCMV-IL-12 or pCMV. Mice were killed 4 days after hydrodynamic injection. Yac1 lytic ability of hepatic mononuclear cells is expressed as the indicated effector and target ratios (E/T ratio). Experiments were done 3 times and representative data are shown. **e** and **f** GKO mice were intrasplenically injected with CT-26 cells and, 1 day later, intravenously injected with whole mononuclear cells, $DX5^+$ cells or $DX5^-$ cells (4.0×10^6/mouse). Two days after CT-26 injection, mice were hydrodynamically injected with either pCMV-IL-12 or pCMV. **e** The levels of serum IFNγ are expressed as mean and SD (n = 6/group). **f** Fourteen days after plasmid injection, mice were killed to examine liver tumor development by measuring liver weight. The results are expressed as mean and SD (n = 6/group). *ND* not detectable. *$p < 0.001$

serum levels of IP-10 and MIG, chemokines downstream of IFNγ, were measured after IL-12 therapy (Fig. 6e). pCMV-IL-12-injected mice showed significant increase in both levels compared with pCMV-injected mice. Significant increase after pCMV-IL-12 injection was also found in NK cell-depleted mice, but not in GKO mice. This result suggests that production of these chemokines was not completely suppressed in NK cell-depleted mice in our experimental condition. Immunohistochemical analysis revealed that tumoral accumulation of CD4-positive cells and CD8-positive cells was observed in pCMV-IL-12-injected mice but not in pCMV-injected mice. On the other hand, similar levels of CD31 expression were observed in tumors of pCMV-injected mice and pCMV-IL-12-injected mice (Fig. 6d). These results suggest that IL-12's anti-tumor effects might be mediated by T-cell accumulating in the tumor rather than anti-angiogenesis.

Discussion

IL-12 is recognized as a master regulator of adaptive type 1, cell-mediated immunity. One major action of IL-12 is its induction of other cytokines, particularly IFNγ. A large amount of evidence has indicated that IL-12 administration leads to IFNγ production from a variety of immune cells, such as T cells [16], B cells [17], NK cells [18] and NKT cells [22]. The relative impact of each immune cell as the source of IFNγ has been controversial. The present study highlighted NK cells as a most efficient producer of IFNγ that is critical for IL-12-induced anti-tumor effects.

Flow cytometric analysis revealed higher in vivo production of IFNγ of NK cells than that of other cell types. The levels of serum IFNγ were around fourfold higher in Rag2 KO mice which only possess NK cells than in wild-type mice. On the other hand, NK-cell depletion in wild-type mice led to twofold reduction of serum IFNγ levels. These data indicate substantial contribution of NK cells in IFNγ production in vivo. Previous research has demonstrated that the specific cellular effects of IL-12 are due mainly to activation of STAT4 [23, 24]. IL-12-induced STAT4 phosphorylation leads to the production of IFNγ [25]. In agreement with these reports, our in vitro analysis showed that, in contrast to STAT1, STAT4 was directly phosphorylated upon IL-12 stimulation, being independent of IFNγ. Of interest is the finding that NK cells express higher levels of STAT4 than non-NK cells, suggesting that NK cells possess an ideal expression profile of STATs for producing IFNγ upon IL-12 stimulation. Indeed, in vitro analysis revealed that NK cells, upon IL-12 exposure, displayed higher levels of IFNγ production as well as STAT4 phosphorylation than non-NK cells. These in vitro

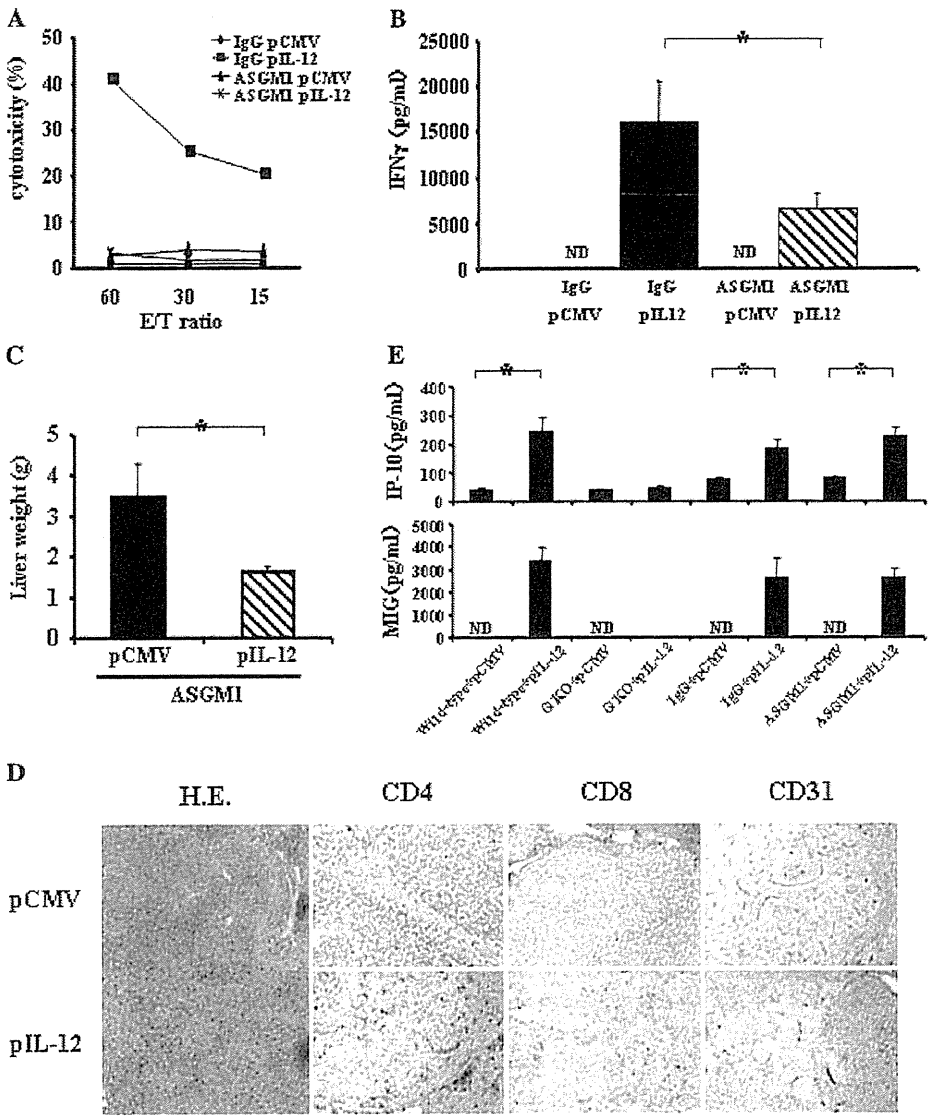

Fig. 6 Anti-tumor effects of IL-12 in NK-cell-depleted mice. Serum IFNγ levels and NK-cell activation. Wild-type mice were intraperitoneally injected with either anti-asialoGM1 antibody (ASGM1) or control IgG, and, 1 day later hydrodynamically injected with either pCMV-IL-12 or pCMV. Mice were killed 4 days after plasmid injection. **a** Yac1 lytic ability of hepatic mononuclear cells is expressed as the indicated effector and target ratios (E/T ratio). Experiments were done 2 times and representative data are shown. **b** The levels of serum IFNγ are expressed as mean and SD ($n = 6$/group). *ND* not detectable. $^*p < 0.005$. Anti-metastatic effects. Wild-type mice were intrasplenically injected with CT-26 cells and, 1 day later and then every 5 days, intraperitoneally injected with either anti-asialoGM1 antibody (ASGM1) or control IgG, and hydrodynamically injected with either pCMV-IL-12 or pCMV 2 days after CT-26 injection. Fourteen days after plasmid injection, mice were killed to examine liver tumor development by measuring liver weight. **c** The results are indicated as mean and SD ($n = 6$/group). $^*p < 0.001$. **d** Representative histology of liver sections analyzed by hematoxylin-eosin staining and immunohistochemistry of CD4, CD8 and CD31. **e** Serum levels of IP-10 and MIG. Wild-type or GKO mice were hydrodynamically injected with either pCMV-IL-12 or pCMV. Wild-type mice were intraperitoneally injected with either anti-asialoGM1 antibody (ASGM1) or control IgG, and 1 day later hydrodynamically injected with either pCMV-IL-12 or pCMV. Four days later, each mice were bled to measure the levels of serum IP-10 and MIG. Results are expressed as mean and SD ($n = 6$/group). *ND* not detectable. $^*p < 0.001$

data are consistent with the in vivo observation that NK cells are efficient producers of IFNγ during IL-12 therapy.

Many studies have demonstrated that IFNγ production is required for the anti-tumor effects of IL-12 [14, 26, 27]. In fact, we have demonstrated that deletion of IFNγ abolished NK cytotoxicity and the anti-metastatic effect of IL-12 therapy in the liver. A large amount of evidence supports the concept that a major action of IL-12 is to promote the differentiation of naïve CD4 + T cells into Th1 cells, which produce IFNγ. Previous research reported that CD4

T-cell depletion caused inhibition of anti-tumor effects. More recent studies have supported a critical role of IFNγ as a third signal for CD8 T-cell differentiation. There have been many reports focusing on IFNγ production from T cells induced by IL-12 for the anti-tumor effect of IL-12 [28]. Segal et al. performed an elegant study showing a critical role of T-cell production of IFNγ in the anti-tumor effect by adoptively transferring T cells into GKO mice in a subcutaneous tumor model [29]. However, apart from this study, little is known about the contribution of each immune cell as a producer of IFNγ in terms of an anti-tumor effect. In our model, T-cell mediated adaptive responses were not required for the anti-metastatic effect of IL-12. More importantly, the anti-metastatic effects of IL-12 were restored in GKO mice by an adoptive transfer of wild-type NK cells. The same number of non-NK cells could not provoke IL-12-induced anti-tumor effects in GKO mice. The present study demonstrated for the first time a potent effect of NK cells on producing IFNγ that was critical for anti-metastatic effect during IL-12 therapy.

Our study showed that the main IFNγ producer of IL-12 was NK cells. So we focused on NK cells which were activated by IL-12 in an IFNγ-dependent manner to examine the cellular mechanism of protection against hepatic metastasis. Many studies have shown the importance of each subset (NK- [12], NKT- [10] and T [9, 30] cells) for anti-tumor effects of IL-12. In the present study, NK cells were sufficient while T cells, B cells, NKT cells were dispensable for IL-12-mediated NK-cell activation and anti-metastatic effects as IL-12 therapy showed Yac1 lytic ability and antimetastatic effects in Rag2 KO mice. On the other hand, NK-cell depletion by a repeated injection of anti-aialoGM1 antibody protected wild-type mice from macroscopic liver metastasis, but did not from microscopic liver metastasis. Thus, although NK cells were required for a full-blown IL-12 anti-tumor effect, other anti-tumor pathways are activated by IL-12 in the absence of NK cells. Serum levels of IP-10 and MIG suggest that production of these chemokines downstream of IFNγ was not suppressed in NK-cell-depleted mice in our experimental condition. When compared with the experiment on GKO mice, accumulation of CD4-positive cells and CD8-positive cells were more evident in NK-cell-depleted mice than in GKO mice (Supplementary Figure). On the other hand, there was no remarkable difference in the expression of CD31 between pCMV injection and pCMV-IL-12 injection. These results suggested that in NK-cell-depleted mice IL-12 may exert anti-tumor effect via T-cell accumulation rather than anti-angiogenesis.

Since the liver contains an abundance of immune cells (especially NK cells) [31], the cytokine-mediated activation of these cells may be a promising approach toward anti-tumor therapy in this organ [32]. IL-12 is a cytokine known to elicit a potent anti-tumor effect in mouse experimental models. However, clinical trials attempted to date were interrupted by fatal adverse effects. Systemic IL-12 therapy has been associated with dose-limiting toxicity [33]. IL-12 induces activation of the pro-inflammatory pathway which causes the complications of high dose cytokine, independent of the action of IFNγ [34]. On the other hand, the levels of immunosuppressive cytokine, for example, TGF-β1 or IL-10 were significantly higher in patients with hepatocellular cancer and colon cancer [35–38]. In particular, TGF-β1 in serum can limit NK-cell IFNγ production [39]. Thus, in patients with advanced disease, IL-12 may not be able to exert its potent anti-tumor immune-effects because IFNγ, which is an important mediator of the IL-12-induced immune response, is less effective in a tumor environment. In the present study, we demonstrated that NK-cell IFNγ production induced by IL-12 was sufficient for the anti-metastatic effect of IL-12 in the liver. Thus, a strategy of efficiently producing IFNγ from NK cells may be important for avoiding toxicity of IL-12 therapy.

IL-12 gene therapy has an advantage to allow local production of the cytokine at the tumor sites with low serum concentration. Studies demonstrated that intratumoral administration of adenovirus encoding IL-12 to animals with different types of carcinoma caused complete tumor eradication and increased long-term survival [40, 41]. Moreover, injection of IL-12-encoding adenovirus in one nodule of liver tumor resulted in regression of distant nodules in the liver [41]. However, in a clinical trial anti-tumor activity of IL-12-encoding adenovirus was only observed in the injected tumor sites, but not in distant tumors [42]. The present study shed light on hydrodynamic transfection of hepatocytes as a promising strategy to eradicate disseminated tumors from whole liver.

In summary, NK cells are not just an effector for innate immunity but a mediator producing IFNγ that is critical for the IL-12 anti-tumor effects. Extremely higher expression of STAT4 may be a basis for efficient production of IFNγ from NK cells.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Absence of invariant natural killer T cells deteriorates liver inflammation and fibrosis in mice fed high-fat diet

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Abstract
Background Invariant natural killer T (iNKT) cells have been suggested to play critical roles in a wide range of immune responses by acting in a proinflammatory or anti-inflammatory manner. Nonalcoholic steatohepatitis (NASH) is a chronic liver disease progressing to advanced cirrhosis and hepatocellular carcinoma. Despite the abundance of iNKT cells in the liver, their role in the pathogenesis of NASH remains obscure. Here, we investigated their role in the development of diet-induced steatosis/steatohepatitis.
Methods We used BALB/c wild-type mice and Jα18-deficient (KO) mice lacking iNKT cells fed either a normal diet or a high-fat diet (HFD). The liver and blood were collected from these mice to examine liver inflammation, steatosis, and fibrosis at the indicated time points.
Results KO mice fed the HFD, compared with control mice fed the HFD, exhibited a clearly higher serum alanine aminotransferase level and a greater number of hepatic inflammatory foci, although there was no significant difference in hepatic lipid retention between these groups of mice. The HFD enhanced hepatic messenger RNA expression of inflammatory cytokines and chemokines in KO but not in control mice. The HFD also increased the proportion of hepatic CD4 T cells and CD8 T cells that composed hepatic inflammatory foci in KO mice, but not in the controls. Prolonged feeding with the HFD augmented liver fibrosis in KO but not in control mice.
Conclusions These findings indicate that iNKT cells play a protective role against liver inflammation progressing to fibrosis, but not against steatosis, enhanced by dietary excess fat, suggesting a key role of these cells in NASH pathogenesis.

Keywords iNKT cells · Nonalcoholic fatty liver disease · Nonalcoholic steatohepatitis · Cytokine · Chemokine

Abbreviations

NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
iNKT	Invariant natural killer T
NK	Natural killer
TCR	T cell receptor
Th	T helper
IFN	Interferon
IL	Interleukin
WT	Wild type
ND	Normal diet
HFD	High-fat diet
KO	Jα18-deficient
ALT	Alanine aminotransferase
RT-PCR	Reverse transcription polymerase chain reaction
H&E	Hematoxylin–eosin
SEM	Standard error of the mean
TNF	Tumor necrosis factor
CCL	Chemokine (C–C motif) ligand
CXCL	Chemokine (C–X–C motif) ligand

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is a spectrum of liver disorders ranging from nonalcoholic steatosis to nonalcoholic steatohepatitis (NASH), which can develop to progressive disease including advanced liver fibrosis and hepatocellular carcinoma [1, 2]. Prolonged overnutrition causes accumulation of free fatty acid and triglycerides within the liver, which is referred to as steatosis. Simple steatosis leads to a predisposition for steatohepatitis, which exhibits inflammatory cell accumulation and fibrosis in the liver in addition to the steatosis [1, 2]. To transform from steatosis to steatohepatitis, several key biological responses such as oxidative stress, mitochondrial dysfunction, endoplasmic reticulum stress, and abnormal cytokine properties have been reported to be required [1–4]. However, the immunological aspect, in particular, that is involved in the development of steatosis/steatohepatitis remains to be fully elucidated.

Invariant natural killer T (iNKT) cells are characterized by the expression of surface markers of natural killer (NK) cells together with a single invariant T cell receptor (TCR) encoded by Vα14-Jα18 in mice and Vα24-Jα18 in humans [5]. These cells are included within the population of T cells expressing NK cell markers, also known as NKT cells [5, 6]. iNKT cells recognize glycolipid antigens presented in association with the major histocompatibility complex class Ib molecule CD1d [5], which is expressed on a variety of cells including dendritic cells, B cells, and stellate cells, as well as hepatocytes in the liver [5, 7, 8]. Following the recognition of antigens via TCR, iNKT cells have the ability to produce the T-helper (Th) 1 cytokine, interferon (IFN)-γ, and the Th2 cytokines, interleukin (IL)-4, -5, and -13, modulating subsequent immune responses [5, 6, 9]. These cells have been shown to play a proinflammatory role in some immune responses and an anti-inflammatory role in other immune responses [5, 6, 9]. iNKT cells most frequently reside in the liver in mice [10, 11]. Although humans appear to have proportionally fewer iNKT cells than mice, human iNKT cells also preferentially reside in the liver [12, 13]. Several lines of evidence indicate that the number of NKT cells is dysregulated in the development of NAFLD. Hepatic iNKT cells or NK1.1+ CD3+/TCRβ+ NKT cells, for instance, have been reported to decrease with the development of steatosis in wild-type (WT) as well as leptin-deficient ob/ob mice [14–17]. A reduced level of peripheral Vα24+ NKT cells has been associated with human NAFLD [18]. On the other hand, CD56+ CD3+ NKT cells have been recently reported to be increased in the livers of patients with NAFLD [19]. Also, the adoptive transfer of NK1.1+ CD3+ NKT cells has been shown to alleviate hepatic steatosis in ob/ob mice [20]. However, the precise role of NKT cells in the pathogenesis of NAFLD has not been investigated in the presence of a deficiency of these cells.

In the present study, we used iNKT cell-deficient as well as WT mice fed either a normal diet (ND) or a high-fat diet (HFD), and examined the role of these cells in the development of HFD-induced steatosis/steatohepatitis. We found that the lack of iNKT cells, together with the HFD, led to liver inflammation, which was characterized by the enhanced gene expression of inflammatory cytokines and chemokines and by T cell accumulation. We also found that prolonged liver inflammation in the absence of iNKT cells developed to liver fibrosis which was strongly enhanced by the HFD. This study delineated an immunoregulatory function of iNKT cells and their key role against liver inflammation progressing to fibrosis exacerbated by an HFD, which might represent a clinical aspect of human progressive NAFLD.

Materials and methods

Animals and animal care

Specific pathogen-free BALB/c WT mice were purchased from CLEA Japan (Tokyo, Japan) as needed. Breeding pairs of BALB/c Jα18-deficient (KO) mice [21, 22] were provided by Drs. Masaru Taniguchi and Ken-ichiro Seino (RIKEN, Yokohama, Japan). The KO mice were confirmed to have no iNKT cells by the use of mouse-CD1d tetramers loaded with α-galactosylceramide in the flow cytometry procedure described below (data not shown). These mice were kept in isolation facilities at the Institute of Experimental Animal Science, Osaka University. They were housed in groups of five in filter cages and were maintained in a temperature-controlled, specific-pathogen-free room on 12-h light and dark cycles with ad libitum access to water and diet as indicated.

Experimental protocol

Male mice used in the experiments were fed an irradiated HFD consisting of 56.7% of the calories from fat (HFD32; CLEA Japan) or an irradiated ND consisting of 14% of the calories from fat (CRF-1; Oriental Yeast, Osaka, Japan), starting from when the mice were 6–8 weeks old. In preliminary experiments, we monitored the body weight of the WT mice and KO mice fed the ND or HFD every 2 weeks after the initiation of feeding, because a gain of body weight usually parallels the level of hepatic steatosis as well as obesity. We did not observe a gain of body weight of more than 25% until 4 weeks after the initiation of feeding. In mice fed the HFD, the body weight gain reached a plateau around 14–16 weeks after the initiation

of feeding. These observations led us to set the time point for estimating liver steatosis and injury and inflammation or liver fibrosis during the course of feeding at week 5 or week 15, respectively. At the end of the indicated periods, the mice were weighed and anesthetized with pentobarbital sodium, and then their abdomens were opened. Following blood sampling via the inferior caval vein, the portal vein and inferior caval vein were cut to enable blood outflow and then the liver was removed, weighed, and processed for further analyses. All animal experimental protocols were approved by the Institute of Experimental Animal Science, Osaka University. To evaluate the levels of liver injury, serum alanine aminotransferase (ALT) activities were measured as previously described [23]. To determine the levels of steatosis, total lipids were extracted from the liver and then triglyceride content was measured as previously described [24].

Flow cytometric analysis

Liver mononuclear cell populations were prepared as previously described [11, 23]. Cell surface staining of the prepared cells was performed as described [11, 23], using the following antibodies or tetramers: fluorescein isothiocyanate-conjugated anti-CD49b (DX5), phycoerythrin-conjugated anti-CD4 (H129.19), peridinin chlorophyll protein-conjugated anti-CD8α (53-6.7), and allophycocyanin-conjugated anti-TCRβ (H57-597) monoclonal antibody, or fluorescein isothiocyanate-conjugated anti-TCRβ, phycoerythrin-conjugated anti-CD4, peridinin chlorophyll protein-conjugated anti-CD45R/B220 (RA3-6B2) monoclonal antibody, and allophycocyanin-conjugated mouse-CD1d tetramers loaded with α-galactosylceramide. All antibodies were purchased from BD Biosciences (San Jose, CA, USA). Mouse CD1d tetramer was obtained from Proimmune (Oxford, UK) and the loading with α-galactosylceramide was performed following the manufacturer's protocol. The stained cells were analyzed with a FACScan (Becton Dickinson, Mountain View, CA, USA), and the data were processed using the CELLQuest program (Becton Dickinson). iNKT cells were detected on electronically gated CD45R/B220- TCRβ+ CD1d-tetramer-reactive cells.

RNA isolation and analysis

Total RNA was isolated from frozen liver tissues by using an RNeasy kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol. Complementary DNA was synthesized from isolated RNA using SuperScript III and random hexamer (Invitrogen, Carlsbad, CA, USA). Real-time reverse transcription polymerase chain reaction (RT-PCR) analysis was performed using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) normalized to beta-actin.

Histological evaluation

The removed liver was partly fixed in 10% formalin for staining with hematoxylin–eosin (H&E), Sirius-Red, or Oil-red-O, or it was immediately embedded in Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo, Japan) and frozen in liquid nitrogen for immunohistochemical staining. Sirius-Red staining was performed to assess liver fibrosis, which was quantified by the extent of the area, using image-analysis software, WinROOF (Mitani, Fukui, Japan). Intracellular lipid was stained with Oil-red-O. To evaluate the infiltration of CD4+ cells or CD8+ cells into the liver, acetone-fixed fresh-frozen tissue sections were immunostained with anti-mouse CD4 (H129.19) or anti-mouse CD8α (53-6.7) monoclonal antibody, respectively, using a VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA, USA) following the manufacturer's protocol. The sections were developed with diaminobenzidine (DAB) substrate (Vector Laboratories) and then counter-stained with hematoxylin. Antibody against CD4 or CD8 was purchased from BD Biosciences.

Statistical analysis

The statistical significance of differences between two groups was determined by applying the Mann–Whitney *U*-test. Statistical significance was defined as $P < 0.05$. All data are shown as mean ± standard error of the mean (SEM).

Results

Lipid accumulation in the liver induced by the HFD was independent of the presence or absence of iNKT cells

To investigate the role of iNKT cells in the development of diet-induced steatosis/steatohepatitis, we fed the ND or HFD to WT and KO mice for 5 weeks. The HFD increased the body weight by around 30% at week 5 in both WT and KO mice, while the ND increased it by around 14% (HFD-fed WT mice 31.6 ± 2.4%, HFD-fed KO mice 29.7 ± 5.6%, ND-fed WT mice 15.5 ± 0.6%, ND-fed KO mice 13.5 ± 1.1%; $n = 5$). The weight gains with the HFD or ND were not significantly different between WT and KO mice. Evaluation of the liver weight at week 5 showed that the HFD-fed WT or KO mice possessed significantly heavier livers than the ND-fed WT or KO mice, respectively, without any significant differences between the WT and KO mice (HFD-fed WT mice 1.95 ± 0.06 g, HFD-fed

KO mice 1.89 ± 0.07 g, ND-fed WT mice 1.52 ± 0.04 g, ND-fed KO mice 1.50 ± 0.06 g; $n = 5$).

We next performed Oil-red-O staining of liver sections from the mice to examine whether the absence of iNKT cells would affect the HFD-induced lipid accumulation in the liver. The staining showed that the HFD, compared with the ND, induced marked lipid retention in hepatocytes in both WT and KO mice (Fig. 1a). Evaluation of the liver triglyceride level demonstrated that the HFD, compared with the ND, clearly induced triglyceride accumulation in the livers of both WT and KO mice, without a significant difference between these groups of mice (Fig. 1b).

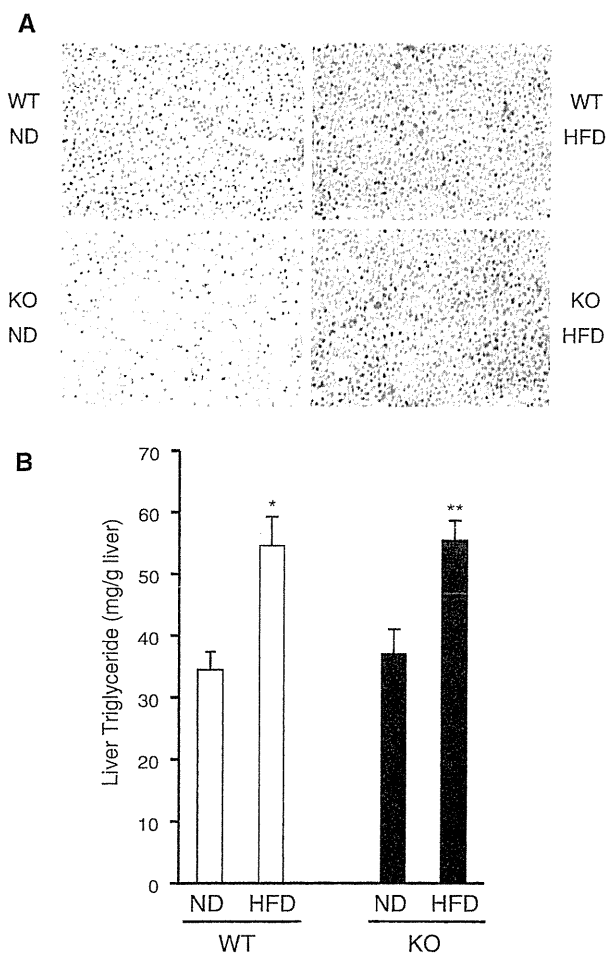

Fig. 1 Lipid accumulation in the liver induced by high-fat diet (*HFD*). Livers were obtained from BALB/c wild-type (*WT*) and BALB/c Jα18-deficient (*KO*) mice fed either a normal diet (*ND*) or an HFD for 5 weeks. **a** Lipid accumulation in liver sections was visualized by Oil-red-O staining. Representative images are shown (×200). **b** Hepatic triglyceride levels were quantified. Data shown are means ± SEM from five mice in each group. Data are representative of more than four independent experiments. $*P < 0.05$ versus WT fed ND. $**P < 0.05$ versus KO fed ND

Collectively, these results suggested that the absence of iNKT cells did not affect the level of HFD-induced steatosis.

HFD augmented liver injury and inflammation in the absence of iNKT cells

To examine the levels of liver injury, we measured ALT activity in serum from WT and KO mice fed the ND or HFD at week 5 after the start of being fed the diets. The serum ALT level in the HFD-fed WT mice (35.8 ± 1.98 IU/l) was significantly higher than that in the ND-fed WT mice (25.2 ± 0.66 IU/l) (Fig. 2a). The serum ALT level in the HFD-fed KO mice (174.8 ± 61.2 IU/l) was also significantly higher than that in the ND-fed KO mice (36.4 ± 7.48 IU/l). It was also higher than that in the HFD-fed KO mice at week 2 (83.3 ± 16.5 IU/l). Of note is the finding that the magnitude of the increase in ALT level at week 5 was clearly much higher in KO (4.9-fold) than in

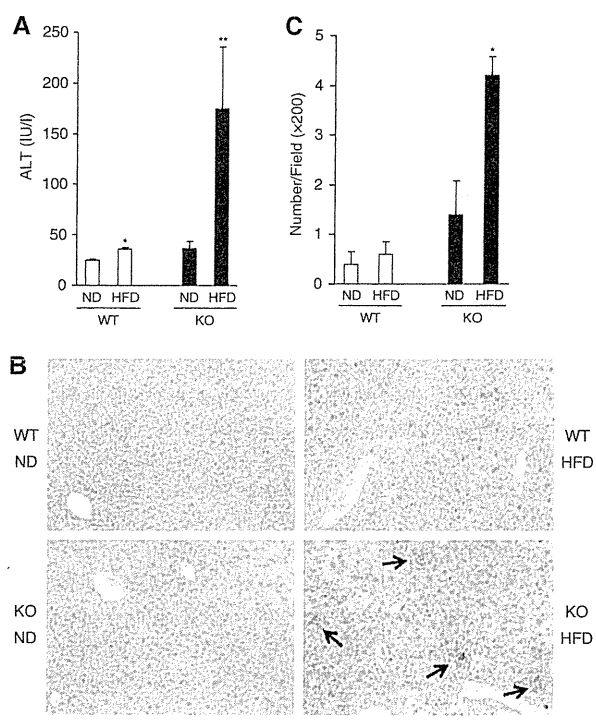

Fig. 2 Liver injury and inflammation exacerbated by HFD in the absence of invariant natural killer T (*iNKT*) cells. Serum and livers were obtained from wild-type (*WT*) and Jα18-deficient (*KO*) mice fed either a normal diet (*ND*) or a high-fat diet (*HFD*) for 5 weeks. **a** Serum alanine aminotransferase (*ALT*) levels were measured. $*P < 0.05$ versus WT fed ND. $**P < 0.05$ versus KO fed ND. **b** Liver tissues were stained with hematoxylin–eosin. Representative images are shown (×200). *Arrows* indicate the inflammatory foci. **c** The numbers of the foci were counted in five different fields per section. $*P < 0.05$ versus KO fed ND. All data shown are means ± SEM from five mice in each group. Data are representative of more than four independent experiments

WT mice (1.5-fold), even though the serum ALT level in the ND-fed KO mice was modestly higher than that in the ND-fed WT mice.

We next conducted histological analyses of liver sections from the mice. H&E staining revealed that the livers from KO mice fed the HFD possessed not only steatotic areas but also scattered inflammatory foci composed of gathering nonparenchymal cells (Fig. 2b). Although inflammatory foci were also observed in the livers from KO mice fed the ND, a larger number of foci could clearly be seen in KO mice fed the HFD than in KO mice fed the ND (Fig. 2c). In contrast, WT mice fed the HFD, as well as those given the ND, showed few inflammatory foci. Taken together, these results indicated that the HFD augmented liver inflammation in KO mice but not in WT mice.

The HFD enhanced hepatic inflammation-related gene expression in the absence of iNKT cells

To understand the underlying mechanisms of the hepatic inflammation induced by the HFD in the absence of iNKT cells, we first examined the levels of several cytokines and chemokines in the livers from mice at week 5 after they had been started on the diets. Real-time RT-PCR analyses revealed that the messenger RNA expression of tumor necrosis factor (TNF)-α, IFN-γ, IL-10, chemokine (C–C motif) ligand (CCL) 2 and 4, and chemokine (C–X–C motif) ligand (CXCL) 9 and 10 were remarkably upregulated by the HFD, compared with the ND, in KO but not in WT mice (Fig. 3), although these values in KO mice fed the ND were modestly higher than those in WT mice fed the ND. In contrast, the messenger RNA expression of IL-4, -5, and -13 did not show any detectable levels in the livers from both WT and KO mice fed either the ND or HFD.

The HFD altered the proportions of subpopulations in liver mononuclear cells from KO mice, but not in those from WT mice

We next examined the phenotype of mononuclear cells in livers from mice at week 5 of feeding. Flow cytometric analyses demonstrated that the proportion of CD4+ TCRβ+ CD4 T cells was lower in KO mice fed the ND than in WT mice fed the ND (Fig. 4a), which might have resulted from a lack of iNKT cells partly composed of CD4+ cells [5]. The proportion of CD49b+ TCRβ− NK cells or CD8+ TCRβ+ CD8 T cells was higher in KO mice fed the ND than in WT mice fed the ND. The HFD did not lead to any significant changes in the proportion of hepatic CD4 T, CD8 T, NK, or iNKT cells in WT mice. In contrast, the HFD induced significant increases in the proportion of hepatic CD4 T cells and CD8 T cells, but not of NK cells, in KO mice. We then examined the distribution of these cells

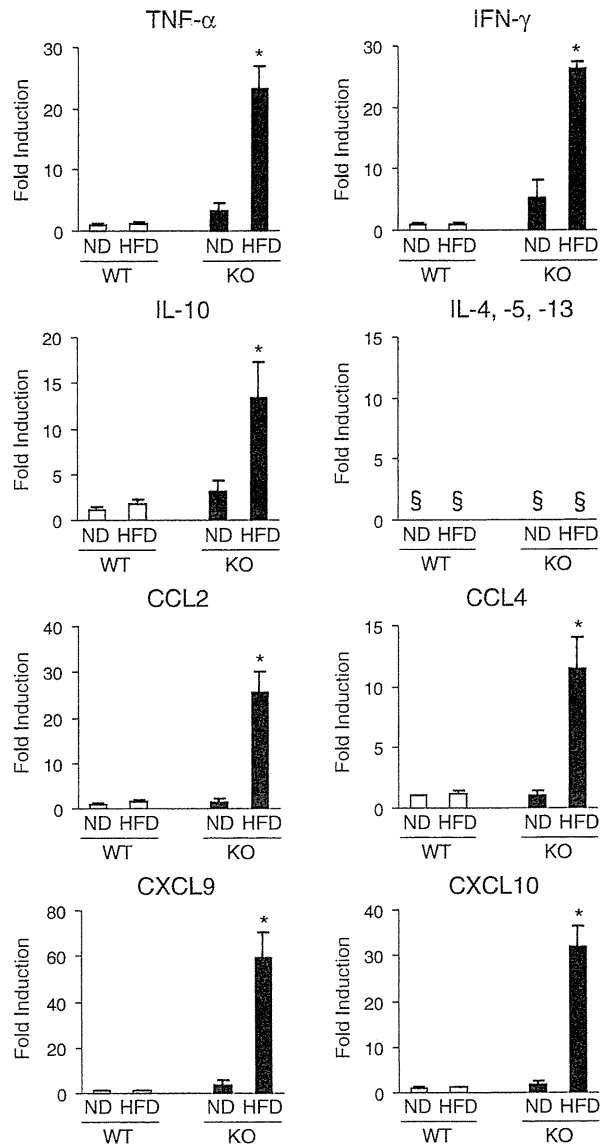

Fig. 3 Inflammatory cytokine and chemokine gene expression in the liver. Liver tissues were obtained from wild-type (*WT*) and Jα18-deficient (*KO*) mice fed either a normal diet (*ND*) or a high-fat diet (*HFD*) for 5 weeks. Liver RNA levels of the indicated genes and beta-actin as a control were analyzed using real-time reverse transcription polymerase chain reaction (RT-PCR). Data are shown as the fold increase of HFD-fed WT, ND-fed KO, or HFD-fed KO compared with ND-fed WT mice, with means ± SEM from five mice in each group. Data are representative of more than four independent experiments. *TNF-α* Tumor necrosis factor alpha, *IFN-γ* interferon gamma, *IL* interleukin, *CCL* chemokine (C–C motif) ligand, *CXCL* chemokine (C–X–C motif) ligand. §, not detected. $^*P < 0.05$ versus KO fed ND

in the liver. Immunohistochemical examination revealed that CD4+ cells and CD8+ cells formed foci surrounding hepatocytes in the livers of KO mice (Fig. 4b), which partly corresponded to the inflammatory foci observed in the

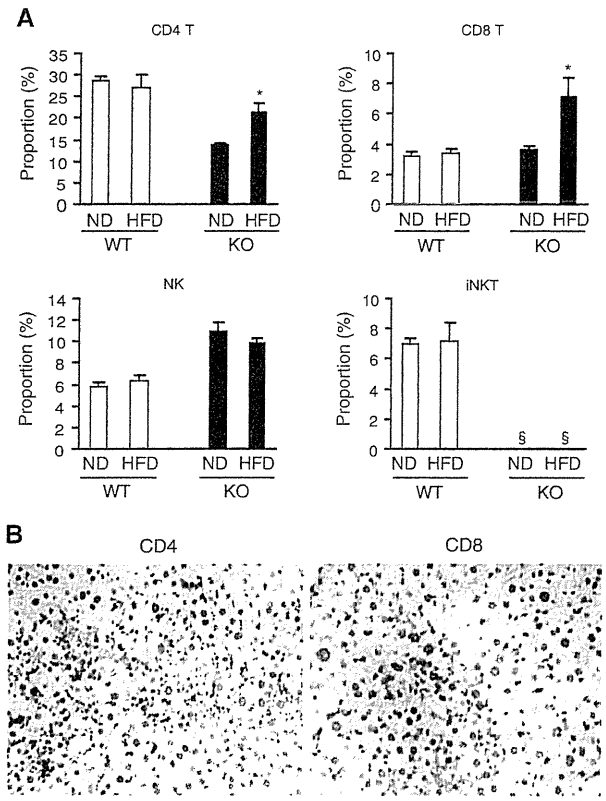

Fig. 4 Involvement of CD4 T and/or CD8 T cells in liver inflammation. Livers were obtained from wild-type (*WT*) and Jα18-deficient (*KO*) mice fed either a normal diet (*ND*) or a high-fat diet (*HFD*) for 5 weeks. **a** Prepared mononuclear cells from the livers were stained with cell markers indicated in "Materials and methods". Proportions of the indicated cell population were analyzed by flow cytometry. Data shown are means ± SEM from five mice in each group. Data are representative of more than four independent experiments. §, not detected. $^*P < 0.05$ versus KO fed ND. **b** Liver sections were analyzed by immunohistochemical staining for CD4- or CD8-positive cells. Representative images are shown (×200)

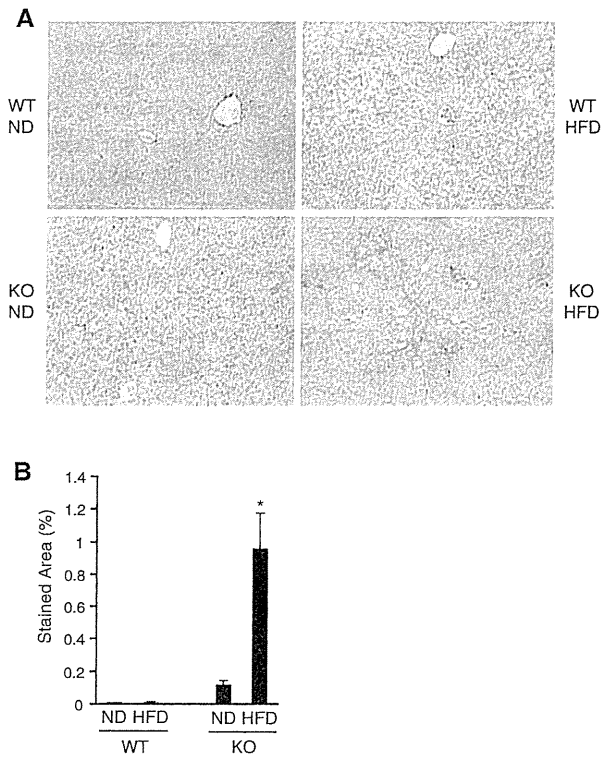

Fig. 5 Liver fibrosis following inflammation in the absence of iNKT cells. Livers were obtained from wild-type (*WT*) and Jα18-deficient (*KO*) mice fed either a normal diet (*ND*) or a high-fat diet (*HFD*) for 15 weeks. **a** Liver tissues were stained with Sirius-Red to assess liver fibrosis. Representative images are shown (×200). **b** The stained areas were evaluated in five different fields per section. Data shown are means ± SEM from five mice in each group. Data are representative of more than two independent experiments. $^*P < 0.05$ versus KO fed ND

H&E-stained liver sections of KO mice. WT mice fed either the ND or HFD did not display such foci consisting of stained cells in the livers. Collectively, these results suggested that CD4 T and/or CD8 T cells played a role in the HFD-enhanced liver inflammation in KO mice.

The HFD led to the development of liver fibrosis in the absence of iNKT cells

Persistent hepatic inflammation causes fibrotic changes in the liver [25]. To investigate whether inflammation with steatosis due to the HFD in KO mice would induce fibrosis in the liver, we fed the ND or HFD to WT and KO mice for a longer period of 15 weeks. H&E staining and Oil-red-O staining showed that KO mice fed the HFD possessed the inflammatory foci, together with lipid retention in the liver, at week 15, as well as showing these findings at week 5, and that WT mice fed the HFD showed lipid retention, but few inflammatory foci, in the liver at week 15 (data not shown). Sirius-Red staining revealed clear fibrosis in the livers from KO mice fed the HFD and also in the livers from KO mice fed the ND, but to a much lesser extent (Fig. 5a). In contrast, the staining showed no obvious fibrosis in the livers from WT mice fed the HFD or in those given the ND. Quantitative analyses to evaluate the stained areas also showed that the HFD-fed KO mice possessed significantly greater areas of hepatic fibrosis than the ND-fed KO mice, while WT mice fed either the ND or HFD had few fibrotic areas (Fig. 5b). Taken together, these results indicated that the HFD led to the development of liver fibrosis accompanied by steatohepatitis in KO mice.

Discussion

An increasing amount of evidence suggests that iNKT cells play a role in immune responses in the liver [12], although

the exact implication of that role is controversial. iNKT cells, for instance, have been reported to play a critical role in animal models of liver injury induced by concanavalin A, α-galactosylceramide, or salmonella infection [26–28], suggesting a proinflammatory role of these cells. On the other hand, iNKT cells have been very recently implicated in the suppression of liver damage in a mouse model of cholestasis [29], suggesting an anti-inflammatory role of these cells. The present study, using iNKT cell-deficient mice fed an HFD, demonstrated that the HFD led to the development of steatohepatitis with fibrosis in the absence of iNKT cells, while the HFD led to steatosis but not steatohepatitis in the presence of these cells. This suggests that iNKT cells play a critical role in suppressing the development of inflammation and fibrosis in the steatotic liver.

Our real-time RT-PCR analyses demonstrated that CCL2, CCL4, CXCL9, and CXCL10 were remarkably upregulated by the HFD in KO mice but not in WT mice (Fig. 3). CCL2 or CCL4 has the ability to attract predominantly Th1 cells via chemokine (C–C motif) receptor 2 or 5, respectively. CXCL9 and CXCL10 also attract predominantly Th1 cells via chemokine (C–X–C motif) receptor 3 [30, 31]. Indeed, Th1 cytokines such as TNF-α and IFN-γ were remarkably upregulated by the HFD in KO mice but not in WT mice. Although IL-10, which is one of the anti-inflammatory cytokines, was also upregulated by the HFD in KO mice but not in WT mice, the upregulation of IL-10 may have counteracted the upregulation of the proinflammatory Th1 cytokines TNF-α and IFN-γ. Our flow cytometric analyses and immunohistochemical analyses showed that the proportions of CD4 T and CD8 T cells were increased (Fig. 4a) and that these cells also accumulated to form foci (Fig. 4b) in the livers of KO mice fed the HFD. Bigorgne et al. [32] reported that HFD-induced obesity in leptin-deficient ob/ob mice rendered hepatic mononuclear cells, particularly CD4 T and CD8 T cells, sensitive to chemokines such as CXCL12 and CXCL13, which attract T cells, suggesting an important role of chemokines in liver inflammation with steatosis. Although the sources of the chemokines upregulated in our model were not clear, these chemokines presumably play an important role in the infiltration of proinflammatory cells in the liver of the KO mice fed the HFD. iNKT cells suppress the production of these chemokines directly or indirectly; thus, they may prevent steatohepatitis induced by an HFD.

The liver can be anatomically exposed to gut-derived contents, such as food antigens and bacterial products, via the portal vein [33, 34]. Once these entities flow into the liver, they can activate a variety of cells in the liver, which may be associated with certain types of liver disease [33, 34]. Gut-derived food-antigens can activate T cells [33] and gut-derived bacterial products can stimulate all resident cells in the liver, such as hepatocytes, Kupffer cells, stellate cells, and dendritic cells, via toll-like receptors [33–36]. Moreover, fat itself, particularly saturated fatty acids, stimulates an immune response in the liver [37, 38]. On the other hand, the liver is an immune-tolerogenic organ, in which immune-suppressive cells may play a critical role to keep this organ immunologically silent [33]. The present study demonstrated that liver inflammation was greatly exacerbated—where CD4 T and/or CD8 T cells infiltrated to form foci surrounding damaged hepatocytes—by an HFD in the absence of iNKT cells. This suggests a suppressive role of iNKT cells in the development of liver inflammation with steatosis. Thus, iNKT cells may play an important role in keeping the liver immunologically silent, and the absence of iNKT cells together with steatosis may elicit a break of hepatic immune tolerance, resulting in the activation of CD4 T and/or CD8 T cells to provoke liver inflammation. Consistent with this speculation is the observation that the absence of iNKT cells, even without steatosis, caused modest liver inflammation.

In conclusion, iNKT cells suppress liver inflammation progressing to fibrosis that is exacerbated by HFD-induced steatosis, thus contributing to the maintenance of immune homeostasis in the liver. This study has shed some light on iNKT cells as immunoregulatory cells and their key role in the pathogenesis of NAFLD.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Factors contributing to antiviral effect of adefovir dipivoxil therapy added to ongoing lamivudine treatment in patients with lamivudine-resistant chronic hepatitis B

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Abstract

Purpose The antiviral effect of adefovir dipivoxil (ADV) added to ongoing lamivudine (LAM) treatment for LAM-resistant chronic hepatitis B (CHB) differs among patients. We investigated clinical factors affecting the response to ADV therapy in LAM-resistant CHB.

Methods The subjects were 75 LAM-resistant CHB patients treated with ADV in addition to LAM. Virological response (VR) was defined as HBV DNA clearance (<2.6 logcopies/ml) at 12 months after the start of ADV therapy. Clinical factors contributing to VR were examined by univariate and multivariate analyses.

Results Lower HBV DNA at baseline and negative hepatitis B e antigen (HBeAg) were significant factors affecting VR in univariate analysis. In multivariate analysis, lower HBV DNA at baseline ($P = 0.005$), negative HBeAg ($P = 0.009$), and higher ALT ($P = 0.036$) were significant independent factors contributing to VR. In HBeAg-positive patients, HBV DNA clearance was more frequently observed during ADV therapy in patients with baseline HBV DNA ≤7.0 logcopies/ml than in those with baseline HBV DNA >7.0 logcopies/ml. By contrast, the link of lower HBV DNA at baseline to better therapeutic response was not evident in HBeAg-negative patients.

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Conclusion In ADV therapy added to ongoing LAM treatment for LAM-resistant CHB, lower baseline HBV DNA and negative HBeAg contributed to a better antiviral effect. Addition of ADV should be done promptly before marked increase in HBV DNA, especially in CHB patients showing LAM resistance positive for HBeAg.

Keywords Adefovir dipivoxil · Lamivudine resistance · Chronic hepatitis B

Introduction

More than 350 million people worldwide are chronically infected with hepatitis B virus (HBV) [1]. Chronic HBV infection can cause liver cirrhosis and hepatocellular carcinoma (HCC), resulting in hepatic disease-related deaths of 500,000 to 1.2 million persons [2, 3]. To prevent disease progression and improve the prognosis of patients with chronic HBV infection, HBV DNA replication must be continuously suppressed as much as possible by antiviral therapy. For this purpose, nucleos(t)ide analogs are currently used for a wide range of patients with chronic HBV infection because of their strong antiviral activities and fewer side effects.

Lamivudine (LAM) is the first approved nucleos(t)ide analog for chronic hepatitis B (CHB) patients, but the increasing incidence of LAM resistance during long-term LAM therapy is a serious problem. The emergence rate of the LAM-resistant virus has been reported to be 24% at 1 year and 70% at 4 years of treatment [4]. Almost all LAM resistance is caused by rtM204V/I mutation occurring in the reverse transcriptase domain of the HBV polymerase gene [5].

To counteract this resistance, adefovir dipivoxil (ADV) was considered as it exerts antiviral effects not only on nucleos(t)ide analog-naïve CHB patients but also on LAM-resistant ones [6–9]. ADV-resistant mutation has been reported to be detected in 11% of patients at 3 years and 29% at 5 years for nucleos(t)ide analog-naïve CHB patients [10]. ADV resistance results from rtA181V/T and/or rtN236T mutation [10]. Either switching from LAM to ADV or adding ADV to LAM has been shown to be effective for LAM-resistant CHB patients. In the case of switching from LAM to ADV, ADV resistance has been reported to appear in 18% of patients at 1 year, which is more frequent than in the case of ADV monotherapy for nucleos(t)ide analog-naïve patients [11]. On the other hand, in the case of ADV administration in addition to LAM, the emergence of resistant virus for both LAM and ADV has been reported to be rare for at least 3 years of treatment [12]. Therefore, ADV therapy added to ongoing LAM treatment is currently accepted as the main therapeutic regimen for LAM-resistant CHB patients rather than a switch from LAM to ADV. However, the antiviral effect of ADV therapy in addition to LAM treatment differs among patients with LAM-resistant CHB.

In this study, we investigated clinical factors influencing the therapeutic efficacy of ADV therapy added to ongoing LAM treatment in LAM-resistant CHB patients.

Patients and methods

Patients

The participating centers were 12 institutions in the Osaka area of Japan (Otemae Hospital, Sumitomo Hospital, Osaka Police Hospital, NTT Nishinihon Osaka Hospital, Higashiosaka City General Hospital, Suita Municipal Hospital, Osaka Rousai Hospital, Kinki Central Hospital, Ikeda Municipal Hospital, National Hospital Organization Osaka National Hospital, Itami City Hospital, and Osaka University Hospital). The subjects were 75 consecutive CHB patients showing LAM resistance. Before the preceding LAM therapy, they all had had hepatitis B surface antigen (HBsAg) for more than 6 months and levels of HBV DNA detectable by the polymerase chain reaction (PCR) method [13]. None of them tested positive for hepatitis C virus antibody or human immunodeficiency virus antibody, nor was there evidence of other forms of liver diseases, such as alcoholic liver disease, drug-induced liver disease, or autoimmune hepatitis.

Anti-HBV treatment

All patients were administered 100 mg of LAM daily. Thirteen (17%) patients had had a history of interferon (IFN) therapy. LAM resistance was judged by detection of rtM204V/I mutation (for 37 patients) or by the existence of virological breakthrough (for 38 patients). Virological breakthrough was defined as the reappearance of detectable HBV DNA of more than 1 log increase in HBV DNA from the nadir on repeated occasions. The median duration of the preceding LAM therapy was 38 (range, 11–83) months. After the emergence of LAM resistance, all patients received 10 mg of ADV daily in addition to ongoing LAM therapy. After the commencement of ADV therapy, liver function and HBV DNA tests were conducted monthly for the first 6 months and every 2 months thereafter. Hepatitis B e antigen (HBeAg) and antibody to HBeAg (anti-HBe) were checked every 2 months. The median follow-up duration of ADV therapy was 22 (range 12–51) months. HBV DNA clearance (<2.6 logcopies/ml) at 12 months after the beginning of ADV therapy was defined as a virological response (VR).

Baseline characteristics of the patients

The baseline characteristics of the patients at the commencement of ADV therapy were as follows. They were 59 males and 16 females, with a median age of 54 (range 27–79) years. Forty-one (55%) tested positive for HBeAg, and anti-HBe developed in 34 patients. The virus was genotyped for 13 patients, all of whom were infected with HBV of genotype C. The HBV DNA ranged from 3.1 to >7.6 (median 7.1) logcopies/ml, and the median ALT level ranged from 15 to 500 (median 105) IU/L. The median levels of total bilirubin and albumin were 0.8 (range 0.4–3.9) mg/dl and 3.9 (range 2.1–4.8) g/dl, respectively. The median platelet counts were 11.7 (range 3.5–25.5) $\times$ $10^4/mm^3$. Of the 75 patients, 27 (36%) showed features of cirrhosis by liver biopsy and/or imaging procedures. Five patients (7%) developed HCC as detected by imaging modalities.

HBV testings

HBsAg, HBeAg, and anti-HBe were examined by chemiluminescent immunoassay. HBV DNA was measured by the PCR-based method (Amplicor HBV monitor, Roche Diagnostics, Tokyo, Japan) [13], with a lower detection limit of 2.6 logcopies/ml. The LAM-resistant rtM204V/I mutation was examined by PCR-enzyme-linked minisequence assay [14]. HBV genotype was determined based on PCR-direct sequencing of portions of core and polymerase genes. The primers used for this study were BF1s (5′-TTT TTC ACC TCT GCC TAA TCA-3′, nt 1821–1841), BR3 (5′-TTC CCG AGA TTG AGA TCT TC-3′, nt 2440–2421), BF6 (5′-CCT CCA ATT TGT CCT GGC TA-3′, nt 350–369), and BR8 (5′-TTG CGT CAG CAA ACA CTT GG-3′, nt 1195–1176) [15, 16].

Statistical analysis

Group comparisons were carried out by the chi-square test, Student's t test and Mann–Whitney's U test. Independent factors contributing to VR during ADV therapy added to ongoing LAM treatment were estimated using multivariate multiple logistic regression analysis in combination with stepwise regression analysis. A P-value of less than 0.05 (two-tailed) was considered to indicate a significant difference. All statistical analyses were performed using the SPSS version 15.0J software (SPSS, Chicago, IL).

Results

Virological and biochemical response to ADV therapy added to ongoing LAM in CHB patients showing LAM resistance

Of the 75 CHB patients showing LAM resistance who underwent ADV therapy added to ongoing LAM treatment, HBV DNA clearance was achieved in 29 (39%) of 75 at 6 months, 35 (47%) of 75 at 12 months, and 34 (72%) of 47 at 24 months. Among the HBeAg-positive patients, HBeAg loss was observed in 8 (20%) of 41 at 6 months, 7 (18%) of 39 at 12 months, and 6 (22%) of 27 at 24 months. As for the biochemical response, ALT normalization ($\leq$40 IU/l) was seen in 57 (76%) of 75 at 6 months, 56 (75%) of 75 at 12 months, and 40 (85%) of 47 at 24 months of treatment.

Pretreatment clinical factors associated with therapeutic response to ADV in addition to LAM treatment

We first investigated pretreatment clinical factors associated with the therapeutic efficacy of ADV added to ongoing LAM treatment by univariate analysis. The baseline characteristics of patients at the beginning of ADV therapy in addition to LAM in the presence or absence of VR are shown in Table 1. Patients showing VR had significantly lower HBV DNA at baseline than patients who did not achieve VR [median 6.3 (range 3.1 to >7.6) vs. 7.3

Table 1 Patient clinical characteristics at the beginning of ADV therapy in addition to LAM in LAM-resistant CHB patients in the presence or absence of virological response (VR)

Clinical characteristics	VR (n = 35)	Non-VR (n = 40)	P value
Gender (male/female)	26/9	33/7	0.386
Age (years)	52 (28–67)	55 (27–79)	0.896
Duration of prior LAM therapy (months)	38 (12–83)	37 (13–64)	0.856
Positive HBeAg	12 (34%)	29 (73%)	0.001
HBV DNA (logcopies/ml)	6.3 (3.1 to >7.6)	7.3 (3.9 to >7.6)	0.002
ALT (IU/l)	106 (16–500)	75 (15–455)	0.136
Total bilirubin (mg/dl)	0.9 (0.4–3.9)	0.7 (0.4–3.9)	0.664
Albumin (g/dl)	4.0 (2.4–4.8)	3.8 (2.1–4.6)	0.351
Platelet count ($\times 10^4/mm^3$)	12.2 (4.8–24.1)	11.5 (3.5–25.5)	0.854
Liver disease (chronic hepatitis/cirrhosis)	20/15	28/12	0.247
Presence of HCC (%)	2 (6%)	3 (8%)	0.757

Continuous variables are expressed as median (range)

Table 2 Baseline factors affecting virological response (logistic regression analysis, stepwise method)

Factors	Category	Odds ratio	95% CI	*P*
Gender	Male/female			NS
Age (years)	By 1 year			NS
Duration of prior LAM therapy (months)	By 1 month			NS
HBeAg	Negative/positive	5.766	1.855–36.62	0.009
HBV DNA (logcopies/ml)	By 1 logcopy/ml	2.362	1.335–5.178	0.005
ALT (IU/l)	By 1 IU/l	1.006	1.000–1.011	0.036
Total bilirubin (mg/dl)	By 1 mg/dl			NS
Albumin (g/dl)	By 1 g/dl			NS
Platelet count ($\times 10^4/mm^3$)	By $1 \times 10^4/mm^3$			NS
Liver disease	Chronic hepatitis/cirrhosis			NS
Presense of HCC (%)	No/yes			NS

CI Confidence interval, *NS* not significant

(range 3.9 to >7.6), $P = 0.002$]. HBeAg was detected in only 12 (34%) of 35 patients with VR, compared with 29 (73%) of 40 patients without VR ($P = 0.001$). Gender ratio, age, duration of preceding LAM therapy, ALT, total bilirubin, albumin, platelet counts, disease severity, and presence of HCC did not differ between VR and non-VR patients.

Factors affecting the therapeutic response to ADV therapy in addition to ongoing LAM were also evaluated by multivariate analysis (Table 2). Eleven pretreatment clinical factors were applied to the analysis as variables. Two factors, lower baseline HBV DNA ($P = 0.005$, odds ratio: 2.362, 95% confidence interval: 1.335–5.178) and negative HBeAg ($P = 0.009$, odds ratio: 5.766, 95% confidence interval: 1.855–36.62), were selected as significant independent factors affecting VR, as was the case for univariate analysis. In addition, higher baseline ALT was also chosen as a significant independent factor ($P = 0.036$, odds ratio 1.006, 95% confidence interval: 1.000–1.011). As for the biochemical response to ADV therapy added to LAM, no pretreatment clinical factors showed a significant relationship with the occurrence of ALT normalization in our 75 LAM-resistant CHB patients.

HBV DNA clearance during ADV therapy in addition to ongoing LAM treatment according to HBeAg status

Next, we investigated HBV DNA clearance during ADV therapy added to ongoing LAM treatment in LAM-resistant CHB patients positive or negative for HBeAg (Fig. 1). In HBeAg-positive patients, HBV DNA was cleared in 8 (20%) of 41 at 6 months, 12 (29%) of 41 at 12 months, and 16 (59%) of 27 at 24 months. On the other hand, HBV DNA clearance was seen in 21 (62%) of 34 at 6 months, 23 (68%) of 34 at 12 months, and 18 (90%) of 20 at 24 months in HBeAg-negative patients. A significant difference ($P < 0.05$) in the frequency of HBV DNA clearance was

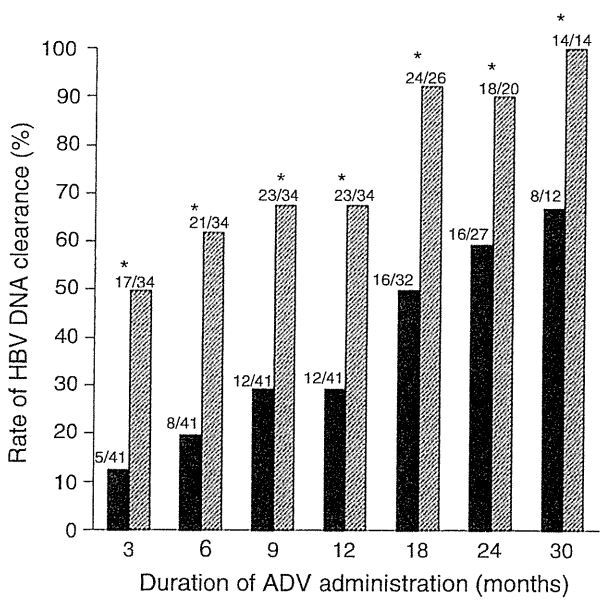

Fig. 1 Rates of HBV DNA clearance in CHB patients positive or negative for HBeAg during ADV therapy in addition to LAM. *$P < 0.05$ between HBeAg-positive and HBeAg-negative patients. *Solid bars* HBeAg-positive patients, *hatched bars* HBeAg-negative patients

observed between HBeAg-positive and HBeAg-negative patients at 3, 6, 9, 12, 18, 24, and 30 months of treatment. Thus, patients negative for HBeAg tended to respond to ADV therapy added to ongoing LAM treatment better than those positive for it in LAM-resistant CHB.

HBV DNA clearance during ADV therapy in addition to ongoing LAM treatment in relation to HBeAg status and baseline HBV DNA

We examined HBV DNA clearance during ADV therapy in addition to ongoing LAM treatment in HBeAg-positive and