

Enhanced Antitumor Response by Divergent Modulation of Natural Killer and Natural Killer T Cells in the Liver

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Abstract

The use of interleukin-18 (IL-18) together with IL-12 induced high levels of IFN- γ in tumor-bearing mice and regression of liver tumors that was abolished in IFN- γ ^(-/-) mice. Natural killer (NK) and NKT cells were the major producers of IFN- γ in the livers of mice treated with IL-18 and/or IL-12. Liver NK cells were significantly increased by treatment with IL-18/IL-12, whereas the degree of liver NKT cell TCR detection was diminished by this treatment. Reduction of NK cells with anti- α GM1 decreased the antitumor activity of IL-18/IL-12 therapy and revealed NK cells to be an important component for tumor regression in the liver. In contrast, the antitumor effects of both IL-18 and IL-12 were further increased in CD1d^(-/-) mice, which lack NKT cells. Our data, therefore, show that the antitumor activity induced in mice by IL-18/IL-12 is NK and IFN- γ dependent and is able to overcome an endogenous immunosuppressive effect of NKT cells in the liver microenvironment. These results suggest that immunotherapeutic approaches that enhance NK cell function while eliminating or altering NKT cells could be effective in the treatment of cancer in the liver. (Cancer Res 2006; 66(22): 11005-12)

Introduction

The liver is a target organ for the formation of metastases by a number of solid tumors, including kidney, colorectal, and breast cancer. At present, only partially effective treatments are available, including surgical resection and liver transplantation (1). Immunotherapy has shown some promise in treating disseminated cancers (2), including liver metastases. A better understanding of interactions within the unique and complex milieu of leukocyte subsets and cytokines in the liver microenvironment may inform more successful immune-based strategies for treatment of malignancies in the liver. Specifically, the liver is a major immunologic organ involved in regulation of tolerance and activation of innate and adaptive immune responses (3, 4). Natural killer (NK) and NKT cells, cellular components of the innate immune system, play critical roles in host defense against viruses (5) and cancer

(6, 7) and are major immune components of the liver microenvironment. Besides killing tumor cells directly, NK and NKT cells are able to rapidly release immunomodulatory cytokines that activate leukocytes of both the innate and adaptive immune systems. Interestingly, CD1d-restricted NKT cells, a subset of NKT cells, have also been previously described to be capable of immunosuppressive functions, in part through the production of interleukin-13 (IL-13; ref. 8). Recently, our lab also found an immunosuppressive role for CD1d-restricted NKT cells in a mouse tumor model (9).

Two cytokines that have antitumor properties and activate NK and NKT cells are IL-18 and IL-12 (10, 11). Besides promoting IFN- γ expression, IL-12 and IL-18 also have similar functional mechanisms that include induction of NK and CD4 cell-mediated cytotoxicity, proliferation of T cells, and activation of NKT cells (reviewed in ref. 12). Because IL-12 and IL-18 activate NK and NKT cells that are present in large numbers in the liver, these cytokines are of potential interest as therapeutic agents against tumors that metastasize to the liver.

Recent studies showed that IL-12 and IL-18 in combination synergistically trigger proliferation and activation of leukocytes, induce IFN- γ production in NK and NKT cells (13, 14), and induce increased antitumor activity when compared with either cytokine alone (15–18). Furthermore, IL-18 and IL-12 synergistically polarize Th₁ responses (19), which should be beneficial for generating antitumor responses. Several studies showed that treatment of NKT cells *in vitro* with IL-18 plus IL-12 (IL-18/IL-12) and their subsequent injection into tumor-bearing mice (16), or coexpression of IL-18 and IL-12 (17) in tumor cells resulted in tumor regression. Other studies showed that coadministration of IL-18/IL-12 had some effects, but severe toxicity limited the effectiveness of this approach (20, 21). In this study, we examine the antitumor potential and effects on liver leukocyte subsets in the liver microenvironment with IL-18/IL-12 treatment of mice. Using this approach, we have been able to show that tumor regression can occur in response to regimens that enhance NK-mediated activity or reduce the number of liver-associated NKT cells, the presence of which directly correlates with increased tumor growth.

Materials and Methods

Reagents. Recombinant murine IL-12 was purchased from PeproTech, Inc. (Rocky Hill, NJ), and recombinant murine IL-18 was kindly provided by GlaxoSmithKline (Upper Merion, PA). Stock aliquots were diluted with HBSS containing 0.1% (v/v) sterile-filtered BALB/c mouse serum and given 5 days a week for 1 to 3 weeks depending on experiment. β -Galactosylceramide(C12) (β -GalCer) used to preferentially deplete NKT cells (22) was purchased from AvantiLipids (Alabaster, AL). Because IL-18 given with IL-12 was previously shown to be toxic (20, 21), we investigated a variety of doses and schedules and were able to determine doses of 0.2 and 0.1 μ g, respectively, that were not toxic while maintaining therapeutic activity.

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Animal care was provided in accordance with the procedures outlined in the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 86-23, 1985).

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Mice and tumor cells. BALB/c and BALB/c severe combined immunodeficient (SCID) mice were obtained from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD). BALB/c-*IFN- γ ^{tm1Ts}* [IFN- γ ^(-/-)] mice with targeted disruption of the *IFN- γ* gene and *CD1^{tm1Gru}* [CD1d^(-/-)] mice that are deficient in both the *CD1d1* and *Cd1d2* genes were originally purchased from The Jackson Laboratory (Bar Harbor, ME) and then bred in our own facility. All mice were maintained in a dedicated pathogen-free environment and used between 8 and 12 weeks of age. Renca, a transplantable spontaneous murine renal carcinoma of BALB/c origin (23), was maintained by serial i.p. passage in syngeneic mice.

Liver tumor model and treatment approaches. Renca cells were injected intrasplenically at a dose of 1×10^5 or 0.5×10^5 cells, 3 or 4 days before initiation of therapy. Splenectomies were done on all mice immediately after tumor injection. Mice were then treated with vehicle control (0.1% normal mouse serum in HBSS), IL-18 (0.2 μ g), and/or IL-12 (0.5 μ g) on days 0 to 4 and 7 to 11. All mice were then euthanized on day 14; livers were harvested; and the number of tumor nodules was counted using a dissecting microscope. To deplete NK cells *in vivo*, anti-asialo GM1 (asGM1) was given i.p. on days -2, -1, and 3 after tumor cell implantation on the liver.

Isolation of liver leukocyte populations. Liver leukocytes were isolated by a modification of a previously described procedure (24, 25). Specifically, the mouse abdomen was opened; the inferior caval vein was cut to enable blood outflow, and the liver was flushed with 10 mL of HBSS through the portal vein using a 10-mL syringe. The liver was removed, and two livers per group were placed in a stomacher bag (Fisher Scientific, Suwanee, GA) along with 25 mL of cold HBSS. The livers were then disrupted for 30 seconds on the medium setting using a stomacher 80 (Seward, West Sussex, United Kingdom), which resulted in a homogenous single cell suspension of liver nonparenchymal cells. Nonparenchymal cells were then collected by centrifugation at $800 \times g$ for 10 minutes at 4°C, washed with cold HBSS, and recentrifuged at $800 \times g$ for 10 minutes at 4°C. The nonparenchymal cell pellet was resuspended in 40% Percoll (Amersham Pharmacia Biotech, Piscataway, NJ), which was then underlaid with 80% Percoll. Percoll solutions were prepared by making $1 \times$ DPBS/Percoll with $10 \times$ DPBS that was diluted with DMEM (Bio Whittaker, Walkersville, MD) to the proper concentration. The cell suspension was gently centrifuged at room temperature for 25 minutes at $1,000 \times g$. The leukocytes were collected at the interface and washed twice in cold HBSS. For *ex vivo* IFN- γ intracellular staining, monensin A (2 μ mol/L; Sigma, St. Louis, MO) was included during the leukocyte isolation process. The isolated liver leukocytes were counted on a Sysmex KX-21 (Roche, Indianapolis, IN) automated cell counter.

Flow cytometric analysis. Flow cytometric analysis was done on liver leukocytes according to a previously described procedure (26). The monoclonal antibodies used were CD4 FITC (clone GK1.5), NK1.1 PE or APC (clone PK136), DX5/CD49b PE, CD8a Biotin (clone 53-67), and CD3 ϵ chain APC (clone 145-2C11; each from PharMingen, Chicago, IL). CD1d tetramer loaded with PBS-57 (α -GalCer analogue) was prepared by the National Institute of Allergy and Infectious Diseases Tetramer Facility.

Intracellular labeling of IFN- γ was done on cells directly isolated from the liver using the Cytofix/Cytoperm kit (PharMingen), according to the manufacturer's suggested protocol using optimally tittered anti-mouse IFN- γ PE (clone XMGL2; PharMingen). During isolation, Monensin A (Sigma) was added to all washes and gradient preparations at 1 μ g/mL to block IFN- γ secretion.

Quantitative real-time PCR analysis. Liver leukocytes were separated from the liver as described above, and genomic DNA for quantitative PCR was isolated using a DNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol for cultured animal cells. PCRs were done on an ABI 7300 real-time PCR system (Applied Biosystems, Foster City, CA). Taqman universal PCR master mix was purchased from Applied Biosystems and used according to the manufacturer's protocol. The primers and Taqman probe for the V α 14 α 18 rearrangement were previously described (27, 28). Real-time PCR data was analyzed using the $\Delta\Delta C_T$ method (28). 18S rRNA endogenous control from Applied Biosystems was used as the internal standard.

Serum cytokine quantitation by cytometric bead array. Mice were euthanized, and blood was obtained by heart puncture. Serum was separated from the RBCs by allowing the RBCs to clot at 4°C for 4 hours then centrifuging the clotted RBCs at $10,000 \times g$ for 10 minutes and collecting the serum phase (clear fraction). Serum (50 μ L) was analyzed for cytokines using the mouse cytometric bead array inflammation kit (BD Biosciences, Immunocytometry Systems, Chicago, IL) on a FACScan flow cytometer affixed with a 488-nm laser (Becton Dickinson Immunocytometry Systems, Mountain View, CA), according to the manufacturer's suggested protocol.

Statistical analysis. *P*s were determined by Welch's *t* test. *P* < 0.05 was considered statistically significant.

Results

IL-18 plus IL-12 induce IFN- γ -dependent regression of established Renca tumor in the liver. In light of the vital role of IFN- γ in tumor immunosurveillance and its necessity for IL-12-induced antitumor activity (29), we examined the relative capacity of IL-18 and/or IL-12 to induce and maintain systemic IFN- γ in mice bearing liver tumors. BALB/c mice were inoculated intrasplenically with 1×10^5 Renca cells on day -3, and treatment with vehicle control, IL-18, and/or IL-12 was begun on day 0 and given i.p. 5 days a week for 3 weeks (three cycles). On days 1, 4, 11, and 18, serum was taken for IFN- γ analysis (Fig. 1A). IL-12 alone induced IFN- γ (68 pg/mL) expression that decreased with daily treatment (Fig. 1A). In contrast, IL-18 and vehicle control induced little to no systemic IFN- γ in Renca-bearing BALB/c mice. Treating mice daily with both IL-18 and IL-12 enhanced IFN- γ expression, which increased up to day 4 (2,902 pg/mL) and remained elevated out to day 18. These findings show that IL-12 and IL-18 strongly synergize for IFN- γ production in mice bearing liver tumors.

Considering the fact that IL-18 and IL-12 have each been reported to have antitumor activities (16, 30), we compared the antitumor effects of each cytokine individually to their effects when given in combination. BALB/c mice were injected intrasplenically with Renca on day -3, and treatment with vehicle control or IL-18/IL-12 begun on day 0 for two cycles as described above. Mice were euthanized on day 14, and tumor nodules in the liver were counted. As expected, a large number of tumor nodules (mean of >300) developed in the livers of vehicle control-treated mice (Fig. 1B). Both IL-18 and IL-12 individually reduced the mean number of tumor nodules to 129 and 54, respectively. However, the combination of IL-18 and IL-12 was significantly better than IL-18 alone (*P* = 0.032) and IL-12 (*P* = 0.047) alone, with a mean of 21 tumor nodules (Fig. 1B).

Given the findings shown above that IL-18/IL-12 synergistically induced IFN- γ and exhibited potent antitumor activity, we investigated the role of IFN- γ in antitumor activity mediated by IL-18/IL-12. Wild-type (WT) and IFN- γ ^(-/-) BALB/c mice were inoculated intrasplenically with Renca and treated with vehicle control or IL-18/IL-12 as described above (Fig. 1C). This study showed that the protective effect of IL-18/IL-12 on mice bearing liver tumors was completely dependent on the presence of IFN- γ because the therapeutic effect of IL-18/IL-12 was completely lost in IFN- γ ^(-/-) mice (Fig. 1C).

Differential modulation of liver NK and NKT cells by systemic IL-12, IL-18, or the combination in mice. Previous studies from our laboratory found that administration of IL-12 to mice modulated the number of liver NK cells (26), whereas the rapid loss of NKT cells after IL-12 treatment has also been noted (31). Hypothesizing that the composition of leukocytes at the disease site may determine the magnitude and outcome of the

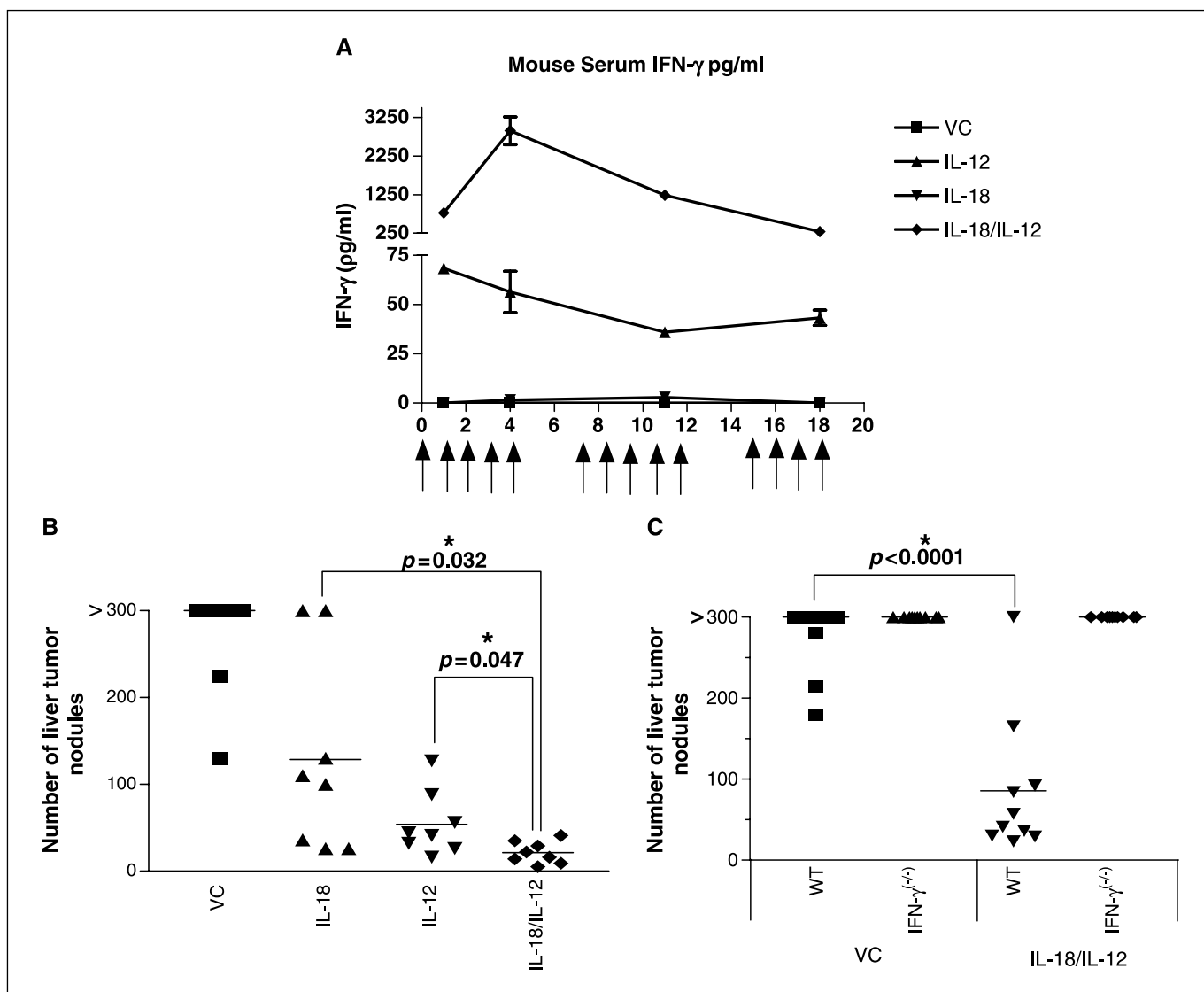


Figure 1. Induction of systemic IFN- γ and its role in IL-18/IL-12 antitumor responses. Renca tumor was implanted in the livers of BALB/c mice intrasplenically. Three days after tumor implantation, mice were treated with vehicle control (VC), IL-18, IL-12, or IL-18/IL-12 on days 0 to 4, 7 to 11, and 14 to 17. **A**, serum from three mice was taken on days 1, 4, 11, and 18 and analyzed for IFN- γ expression by cytometric bead array. **B**, on day 14, livers from eight mice were harvested, and tumor nodules were counted. **C**, on day 17, livers from 10 WT or $IFN-\gamma^{-/-}$ (GKO) mice were harvested, and tumor nodules were counted.

antitumor response, we examined the ability of IL-18/IL-12 to modulate the number of NK and NKT cells in the liver microenvironment of BALB/c mice. To this end, mice were treated daily with vehicle control, IL-18, and/or IL-12, and liver leukocytes were isolated for NK and NKT cell enumeration at 24 and 96 hours. When compared with the vehicle control group, treatment of mice with IL-12, IL-18, or the combination of IL-18 and IL-12 increased the number of liver NK cells [DX5⁽⁺⁾CD3 ϵ ⁽⁻⁾] by >13-, 7-, and 3-fold, respectively, by 96 hours (Fig. 2A). Conversely, all treatments rapidly (24 hours) decreased the detection of liver NKT cells TCR [CD1d tet⁽⁺⁾]. Whereas IL-18/IL-12 sustained a >50% reduced detection of NKT cell TCR, this level returned to baseline by 96 hours with IL-18 or IL-12 single-agent treatment.

A recent study showed that the TCR on NKT cells was down-regulated following TCR engagement (27). To verify that NKT cells were actually reduced in the liver subsequent to IL-18/IL-12 treatment and not simply down-regulating their surface TCR,

quantitative real-time PCR analysis of rearranged V α 14-J α 18 TCR α chain was done on genomic DNA isolated from liver leukocytes of untreated CD1d^(-/-) (negative control) or BALB/c mice treated with vehicle control or IL-12/IL-18 for 24 hours. Using this approach, we found a pronounced reduction of NKT cells at 24 hours in mice treated with IL-18/IL-12 that was confirmed by quantitative real-time PCR relative expression of V α 14-J α 18 TCR α chain (Fig. 2B).

Because tumors likely influence their microenvironment, we analyzed the modulation of NK and NKT cells by IL-18/IL-12 using a DX5 and CD1d tetramer loaded with PBS-57 (α -GalCer analogue) in tumor-bearing mice. Renca tumor was injected via the intrasplenically route into BALB/c mice to selectively grow tumor foci in the liver. The tumors were established for 4 days, and then mice were treated daily with vehicle control or IL-18/IL-12 on days 0 to 3. On day 4, leukocytes were isolated from the liver for flow cytometric evaluation of NK- and CD1d-restricted NKT cells

(Fig. 2C). Treatment with IL-18/IL-12 induced a 57% increase in liver NK cells in tumor-bearing mice at day 4, whereas a 75% decrease in the detection of CD1d tetramer⁺ cells (NKT cells) was observed when compared with the vehicle control-treated mice. Thus, treating mice with the combination of IL-18 and IL-12 resulted in enhanced numbers of liver NK cells but decreased the detectable number of NKT cells.

Controlled expression of IFN- γ in liver NK, NKT, and T cells following treatment of mice with IL-18 and/or IL-12. Several factors derived from the data outlined above and presented in the general scientific literature suggested to us that a careful analysis of IFN- γ production by NK, NKT, and T cells might enhance our understanding of the complex immune milieu in the liver as it relates to regulation of antitumor activity. Specifically, (a) IFN- γ is known to be a key immune mediator in several successful immunotherapeutic strategies (32), including the reduction in liver tumor nodules by IL-18/IL-12 reported above (Fig. 1C). (b) IL-18/IL-12 is known to induce IFN- γ in a synergistic manner (13, 32). (c) And as shown in Fig. 1A, IL-18/IL-12 synergistically induces IFN- γ in mice bearing implanted liver tumors. Therefore, mice were treated with IL-18 and/or IL-12 or vehicle control, and total leukocytes were isolated from the liver and analyzed for the presence of intracellular IFN- γ . As expected, IL-18 and IL-12 given

together induced the highest level of intracellular IFN- γ in total liver leukocytes at both 5 and 24 hours (Fig. 3A). Furthermore, IL-18/IL-12 provoked an elevated level of IFN- γ expression on a per cell basis than did either cytokine alone, based on a log increase in brightness of staining for intracellular IFN- γ in these cells (data not shown). Interestingly, IL-18 induced intracellular IFN- γ (\approx 6%) more rapidly (5 hours) in total liver leukocytes than did IL-12 (\approx 3%). However, the IL-12-induced intracellular IFN- γ (\approx 5%) continued to increase out to 24 hours in total leukocytes, whereas intracellular IFN- γ induced by IL-18 returned to baseline by this time point. Thus, IL-18 and IL-12 synergize to result in both a rapid induction and sustained high levels of IFN- γ expression in liver leukocytes that could not be attained using either cytokine alone.

These results caused us to investigate the relative subset contributions to both early induction and the overall persistence of IFN- γ production in the liver. Treating BALB/c mice once with IL-18 caused 35% of the liver NK cells and 12% of the NKT cells to express IFN- γ at 5 hours, and this incidence fell to baseline for both cell types by 24 hours (Fig. 3B). In contrast, IL-12 induced IFN- γ production in 15% of liver NK cells at 5 hours, and although the percentage of cells expressing IFN- γ remained at 15% at 24 hours, the number of liver NK cells also increased resulting in a 2.5-fold increase in the number of NK cells expressing IFN- γ . IL-12 also

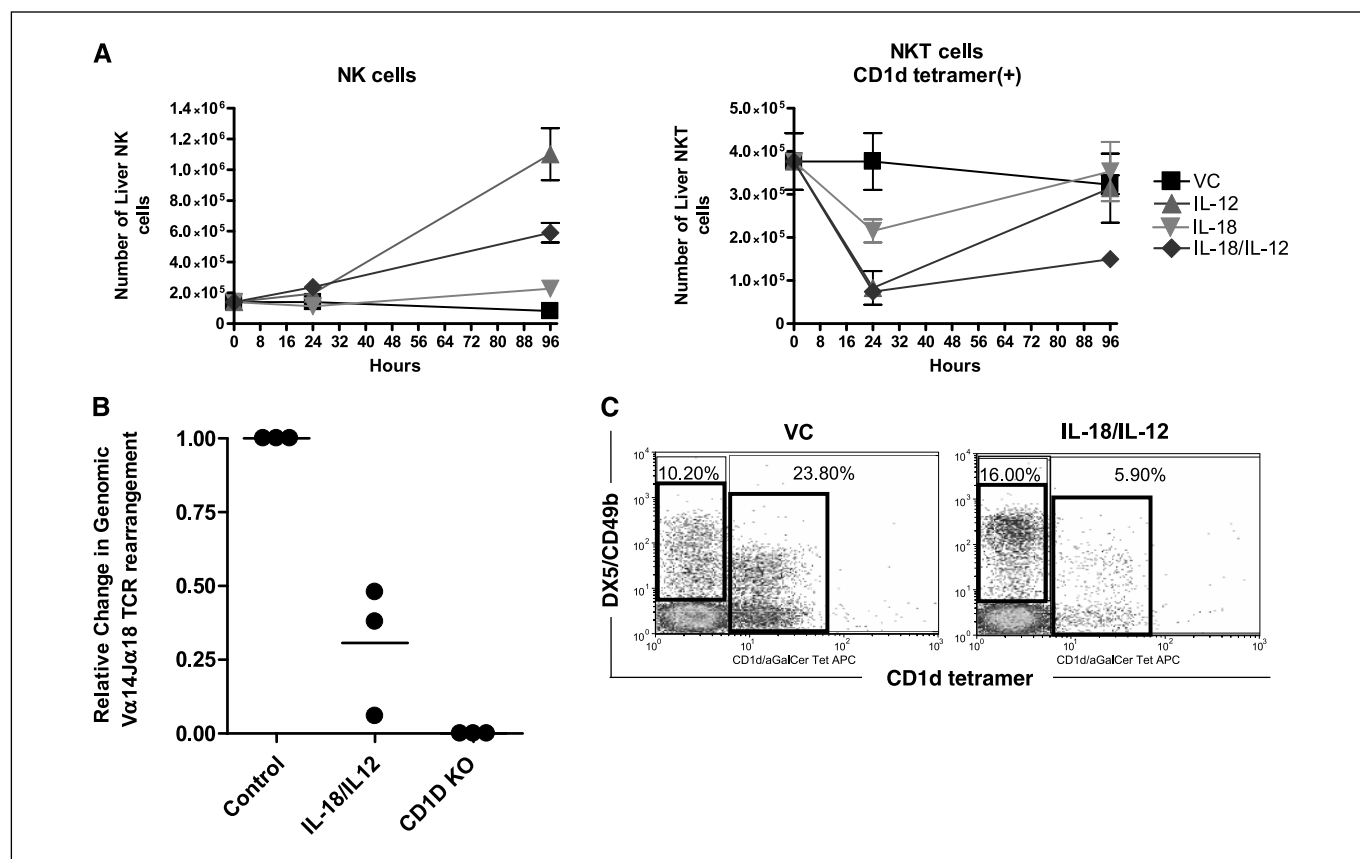


Figure 2. Modulation of liver NK and NKT cells following i.p. administration of IL-18 and/or IL-12. **A**, BALB/c mice were treated with vehicle control, IL-18, and/or IL-12 daily. At 24 or 96 hours, leukocytes from the liver were isolated, and NK and NKT cells were quantitated by flow cytometric analysis using CD3e⁺ and anti-DX5 NK cells or CD1d tetramer⁺ for NKT cells. NK and NKT cell numbers per liver were determined by multiplying the total number of leukocytes by the percentage of each subset. Points, mean of three separate experiments (each experiment used two or three groups of two mice); bars, SE. **B**, genomic DNA, for quantitative real-time PCR analysis of V α 14-J α 18 TCR rearrangement, was extracted from leukocytes isolated from the liver of 8 to 15 CD1d^{-/-} BALB/c and WT BALB/c mice treated with vehicle control or IL-18/IL-12 for 24 hours. Points, mean of three separate experiments; bars, SE. **C**, BALB/c mice bearing Renca tumor on their liver were treated daily for 4 days with vehicle control and IL-18/IL-12. Leukocytes were isolated, and the percentage of NK and NKT cells was determined by staining cells with DX5/CD49b and CD1d/ α -GalCer tetramer. Representative experiment of two mice per group that was repeated four times.

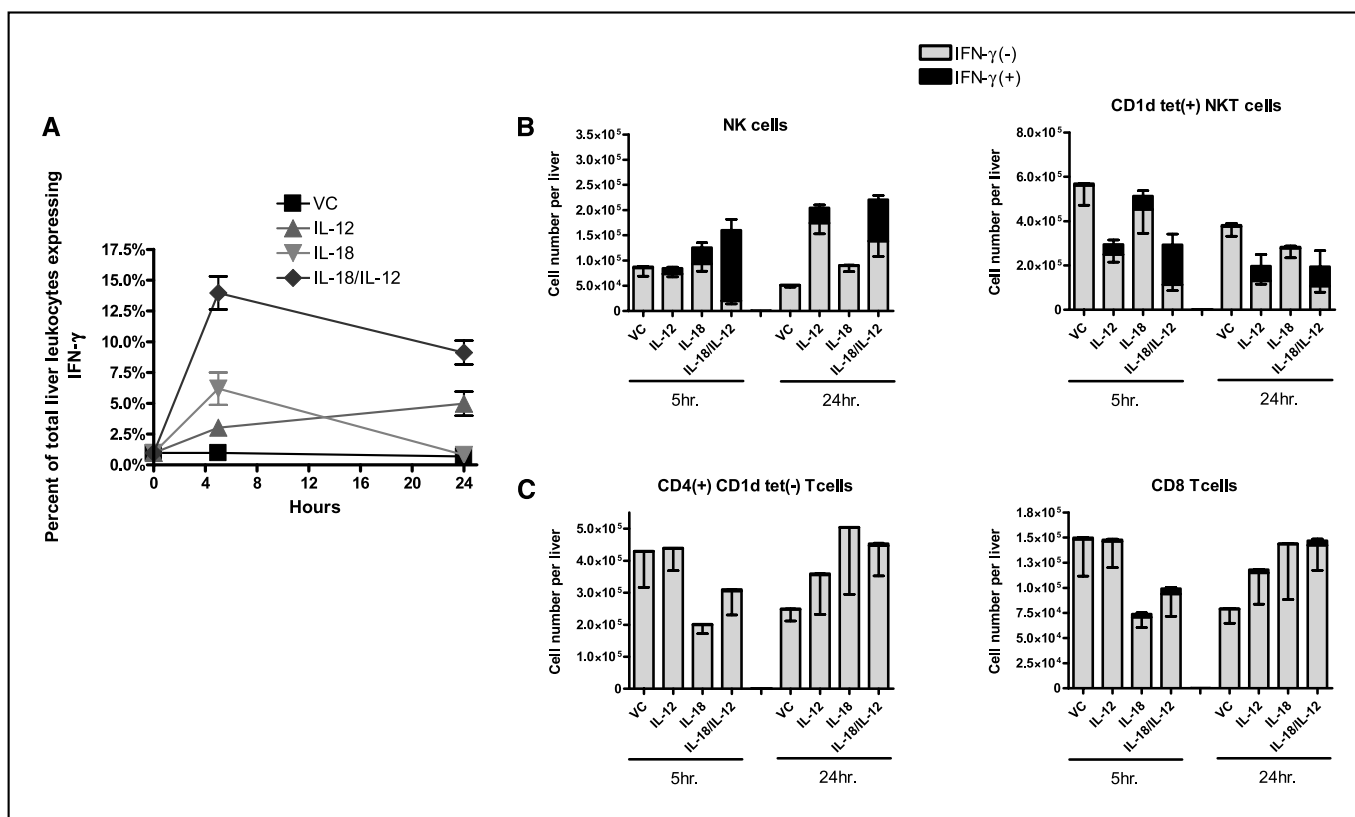


Figure 3. Induction of IFN- γ expression in liver NK and NKT cells following IL-18 and/or IL-12 activation. BALB/c mice were treated once with vehicle control, IL-18, and/or IL-12, and leukocytes were isolated at 5 or 24 hours. A, intracellular IFN- γ expression of total liver leukocytes was examined by flow cytometry. B and C, liver NK cells [DX5⁽⁺⁾ and CD3 ϵ ⁽⁻⁾], NKT cells [CD1d tetramer⁽⁺⁾], CD4 T cells [CD4⁽⁺⁾ and CD1d tetramer⁽⁻⁾], and CD8 T cells were examined for expression of IFN- γ . Gray area, number of cells negative for intracellular IFN- γ ; black area, number of cells expressing IFN- γ . Columns, mean total number of the cell subset of two separate experiments (each experiment used three groups of two mice); bars, SE. Up bar, SE of cells that are IFN- γ ⁽⁺⁾; down bar, SE of cells that are IFN- γ ⁽⁻⁾.

induced IFN- γ expression in 15% of liver NKT cells at 5 hours and 33% at 24 hours. Remarkably, treating mice with the combination of IL-18/IL-12 rapidly induced 88% of the NK cells to express IFN- γ at 5 hours, which was 333% and >1,000% higher than the total number of NK cells expressing IFN- γ following IL-18 or IL-12 treatments, respectively, at 5 hours. By 24 hours, 38% of an expanded NK cell population still expressed IFN- γ following IL-18/IL-12 treatment. IL-18/IL-12 also induced IFN- γ in NKT cells at 5 hours (62%) and at 24 hours (46%), and as with IL-12, the detection of this population with CD1d tetramer was reduced. Thus, IL-18/IL-12 induced a rapid and acute increase in IFN- γ expression in both NK and NKT cells over that induced by either of these cytokines alone. Much of the increased IFN- γ expression in NK cells and some in NKT cells were sustained with IL-18/IL-12, whereas the degree of NKT cell TCR staining was reduced by 24 hours.

In contrast, T cells expressed insignificant levels of IFN- γ in the liver following IL-18/IL-12 treatment at the time points we examined (Fig. 3C).

IL-18/IL-12 early antitumor activity in the liver was mediated by NK cells but not T cells. To define the role of T cells in mediating IL-18/IL-12 antitumor activity in the liver, we compared WT mice with SCID mice that are deficient in T cells. Renca tumor was seeded intrasplenically, and the tumors were allowed to establish for 3 days, before a two-cycle treatment regime of vehicle control or IL-18/IL-12 described above. A significant drop in the number of tumor nodules were noted in SCID mice treated

with IL-18/IL-12 when compared with vehicle control (Fig. 4). However, no significant difference in the number of liver tumor nodules was noted between WT and SCID mice treated with IL-18/IL-12. This study shows that early antitumor activity of IL-18/IL-12 is not dependent upon T cells.

Next, we examined NK cells because IL-18/IL-12 increased their numbers in the liver, and this finding is consistent with a role for NK cells in antitumor activity. To test this hypothesis, mice bearing Renca in the liver were treated with anti-asGM1 as described in Materials and Methods. Treating mice with anti-asGM1 decreased NK cells by 50% in the liver (Fig. 5A). This reduction in NK cells corresponded to a significant increase in the number of liver tumor nodules in mice treated with vehicle control and IL-18/IL-12 when compared with mice that received normal rabbit serum (Fig. 5B). Thus, even a partial reduction in NK cell numbers was capable of diminishing the intrinsic and IL-18/IL-12-induced antitumor activity in the liver.

Selective reduction or elimination of CD1d-restricted NKT cells decreases liver tumor nodules. NKT cells can sometimes play a pivotal role in host defense against tumors (6, 33), but in some settings, a subset of NKT cells can also inhibit immunosurveillance of cancer (8, 9). Results shown above show that IL-18/IL-12 reduced the detectable number of NKT cells in the liver (Fig. 2A and C). Interestingly, the loss of CD1d tetramer staining as induced by IL-18/IL-12 also coincided with the most pronounced antitumor effects (Fig. 1B). These results raise the possibility that this cytokine-induced alteration of NKT cell detection may be

beneficial for the host's ability to eliminate tumor in the liver. To test this hypothesis, β -GalCer, which we have previously shown to preferentially reduce the detection of CD1d-restricted NKT cells without activating them (22), was given to BALB/c mice bearing Renca liver tumors before the delivery of IL-18 or IL-12. We speculated that if CD1d-restricted NKT cells had a regulatory or inhibitory function, their removal would enhance the antitumor activity in mice. Consistent with this hypothesis, we found that the reduction in the detectable number of CD1d-restricted NKT cells with β -GalCer in the vehicle control group resulted in a significant reduction in liver tumor nodules ($P = 0.038$) compared with vehicle control group in WT mice (Fig. 6A). Similarly, significantly fewer tumor nodules were observed when IL-18 was combined with β -GalCer ($P = 0.024$) compared with IL-18 without β -GalCer treatment. Thus, a relatively inefficient antitumor regimen of IL-18 treatment alone could be substantially improved by the removal of CD1d-restricted NKT cells. Finally, IL-12 induced an 86% reduction of liver nodules over vehicle control that was not enhanced by the addition of β -GalCer (Fig. 6A). The absence of an effect by β -GalCer is most likely because IL-12 in and of itself rapidly and efficiently depletes most of the NKT cells from the liver as shown in Fig. 2C; thus, any NKT-depleting effect of β -GalCer on the small residual population of NKT cells would be expected to be inconsequential.

These results strongly support the hypothesis that endogenous CD1d-restricted NKT cells in the liver serve to inhibit spontaneous or cytokine-induced antitumor responses. However, from these studies, it is unclear if β -GalCer is reducing NKT cells or simply causing the down-modulation of their TCR (27, 28). To definitively delineate the role of CD1d-restricted NKT cells in IL-18- and/or IL-12-mediated antitumor activity in the liver, we used CD1d^(-/-) mice that are deficient in NKT cells to assess tumor progression in the liver and then studied the ability of IL-18 or IL-12 to eliminate established tumors. We speculated that in the complete absence of CD1d-restricted NKT cells, the effects of IL-12 or IL-18 would be further improved. Significantly fewer tumor nodules were observed in the livers of untreated CD1d^(-/-) mice compared with WT mice

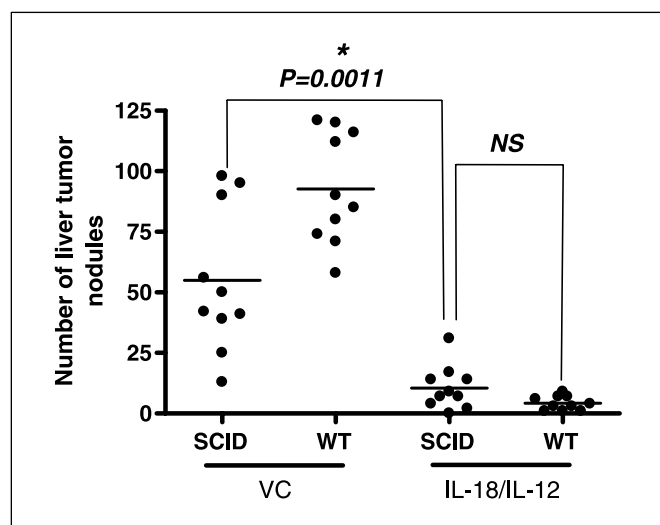


Figure 4. Role of T cells in the antitumor activity of IL-18/IL-12 in the liver. SCID or WT BALB/c mice received Renca tumor intrasplenically, and 3 days later, mice were treated with two cycles of vehicle control or IL-18/IL-12. Seventeen days after tumor implantation, livers were harvested, and tumor nodules were counted.

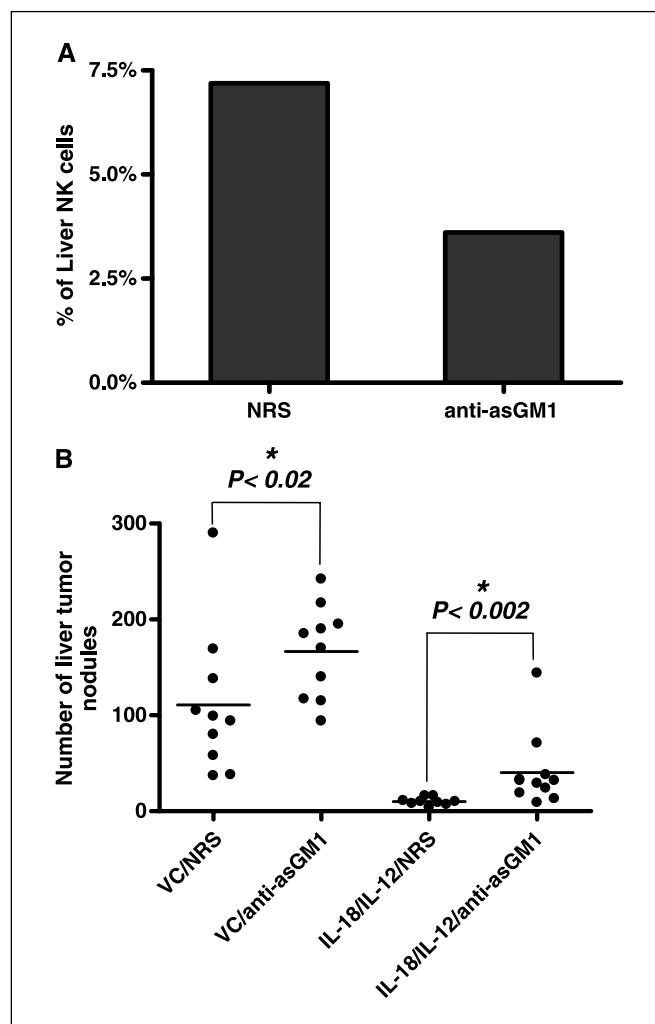


Figure 5. Anti-asGM1 administration reduces the antitumor activity of IL-18/IL-12 therapy in mice bearing experimentally established liver metastases. **A** and **B**, cohorts of 12 BALB/c mice per group were pretreated with normal rabbit serum (control) or asGM1 to deplete NK cells concurrent with intrasplenic implantation of Renca tumors. Three days after tumor implantation, **(B)** livers from two mice were harvested, and NK cells depletion was monitored, or **(C)** mice were given two cycles of i.p. administration of vehicle or IL-18/IL-12. On day 17 before tumor implantation, livers were harvested, and tumor nodules were counted. Columns/points, mean of 10 mice in each group, which was representative of three experiments.

($P = 0.043$), and both IL-18 and IL-12 treatments exhibited significantly more antitumor activity in CD1d^(-/-) mice than in WT mice ($P = 0.013$ and $P = 0.018$, respectively; Fig. 6B). These findings clearly show that CD1d-restricted NKT cells normally resident in the liver impair or counteract the ability of spontaneous and cytokine-induced antitumor mechanisms.

Discussion

We report here that administration of IL-18/IL-12 greatly reduced the number of tumor nodules in a mouse model of established liver tumors. The mechanism for this potent antitumor activity was (a) dependent on synergistic induction of systemic IFN- γ in tumor-bearing mice, (b) associated with an increase in the number of effector liver NK cells, and (c) associated with a decrease in the detectable levels of NKT cell TCR that revealed an inherent

inhibitory activity of these cells for spontaneous and cytokine-induced antitumor responses in the liver.

Several studies have revealed the importance of IFN- γ in tumor immune surveillance (34, 35). Our laboratory and others have reported that IFN- γ production is an important component in a variety of cytokine therapies against tumors (36–38). In the present report, we also found that IFN- γ production is essential for IL-18/IL-12-induced antitumor activity. However, Portielje et al. showed that IFN- γ levels declined with repeated administration of IL-12 to cancer patients (39). We also found that repeated administration of IL-12 to tumor-bearing mice induced an early peak of IFN- γ at 24 hours that was quickly attenuated. Although others showed that

IL-18 given with IL-12 synergistically induced IFN- γ (13, 14), we show in this report that IL-18/IL-12 synergistically induced IFN- γ production in tumor-bearing mice that, unlike with IL-12 alone, was augmented by repeated administration reaching a peak at day 4. Additionally, unlike with IL-12 alone, IL-18/IL-12 coadministration maintains elevated IFN- γ levels for at least several days. Therefore, IL-18/IL-12 therapy is able to maintain high levels of IFN- γ even in a setting of a substantial tumor burden in the liver. Several studies showed that patients with advanced cancer lose the capacity to produce IFN- γ , and clinical responses can sometimes be correlated with the ability to maintain IFN- γ levels (40–42). Thus, therapies that maintain systemic and perhaps local IFN- γ levels, such as IL-18/IL-12 combination treatment, might be particularly effective against established tumors.

Because NK cells have a well-known role as effector cells against tumors (43), it was not surprising that selectively reducing their numbers caused a significant loss of antitumor activity in response to IL-18/IL-12 therapy. Furthermore, we show that NK cells are a significant source of IFN- γ following IL-18/IL-12 therapy, and reducing these cells diminishes a source of IFN- γ , which is a key mediator important for both innate and adaptive cancer therapies. Our data indicated that T cells did not express detectable levels of IFN- γ following early treatments with IL-18/IL-12 and presumably contributed little to this combination therapy. This conclusion was supported by our experiment using SCID mice that showed potent early IL-18/IL-12 antitumor activity in the liver. However, we would not rule out their involvement in long-term IL-18/IL-12-based therapeutic regimens.

In contrast to an increase in NK cell numbers with IL-18/IL-12 treatment of mice, this treatment caused a rapid decrease in the level of detectable NKT cell TCR in the liver. Like NK cells, NKT cells are also significant producers of IFN- γ in response to IL-18/IL-12 treatment. However, unlike NK cells, reducing the degree of detectable NK-T cells or using mice deficient in these cells caused a more robust antitumor activity that was enhanced with cytokine treatment. This finding suggests intrinsic suppressive activity by NKT cells supplants their ability to produce IFN- γ in IL-18/IL-12 therapy. Nonetheless, NKT cells are a source of early beneficial IFN- γ following IL-18/IL-12 treatment before their numbers are reduced in the liver. Thus, an immunosuppressive role for NKT cells was found in our tumor model, and their absence in CD1d KO mice or activation and subsequent reduction with IL-18/IL-12 therapy eliminates this suppression.

The location of the liver as a junction between the blood stream and the gut presents a unique challenge of preventing disease while maintaining tolerance to foreign gut antigens (food, commensal, bacteria, etc.). NKT cells seem to be designed to take on this challenge by playing both a suppressive role (8, 9) as well as an effector role in disease (5, 6, 44). They are capable of expressing both pro- and anti-inflammatory cytokines (33). Furthermore, missing or dysfunctional NKT cells are implicated in various autoimmune diseases (44, 45). We show NKT cells rapidly express IFN- γ before the use of IL-18/IL-12 rapidly reduces their numbers in the liver. These data taken together suggest NKT cells act as immune sentinels holding the local immune system at bay, but once properly activated, call into action the immune system before reducing their numbers.

In conclusion, we have shown that IL-18/IL-12 has potent antitumor activity in the liver that is not observed by using either agent alone. We show that IL-18/IL-12 synergistically induced elevated systemic levels of IFN- γ that was maintained in tumor

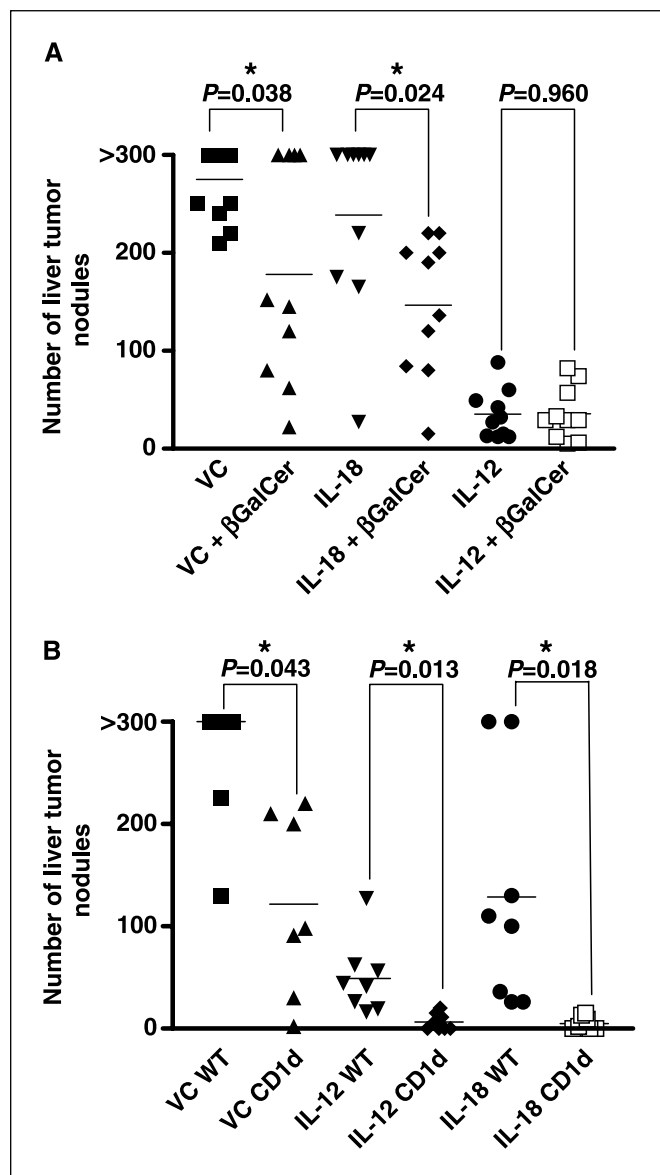


Figure 6. β -GalCer-treated or CD1d^{-/-} mice have enhanced antitumor activity following IL-18 and/or IL-12 treatment. *A* and *B*, cohorts of 10 mice per group were (A) pretreated with β -GalCer to reduce NKT cells before tumor implantation, or (B) CD1d^{-/-} mice deficient in NKT cells were inoculated intrasplenically with Renca. Three days after tumor implantation, mice received daily i.p. administration of vehicle control, IL-18, or IL-12 for two cycles. On day 17 before tumor implantation, livers were harvested, and tumor nodules were counted.

bearing mice. Furthermore, we showed that liver NK and NKT cells are the principal source of a protracted expression of IFN- γ in mice treated with IL-18/IL-12. Importantly, treating mice with IL-18/IL-12 induces an increase in liver effector NK cells and a decrease in immunosuppressive liver NKT cells. This result suggests that immunotherapeutic approaches that preferentially stimulate NK cells, whereas selectively removing NKT cells may be effective in treating tumors in the liver.

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