

NKT Cell Exacerbation of Liver Metastases Arising from Melanomas Transplanted into Either the Eyes or Spleens of Mice

Wanhua Yang, Haochuan Li, Elizabeth Mayhew, Jessamee Mellon, Peter W. Chen, and Jerry Y. Niederkorn

PURPOSE. To explore the role of natural killer T (NKT) cells in the development of liver metastases in mice harboring intraocular melanomas.

METHODS. Cells derived from the cutaneous B16 melanoma cell line (B16LS9) were transplanted either into the vitreous body or under the spleen capsules of wild-type C57BL/6 mice and NKT-cell-deficient $J\alpha 18^{-/-}$ and $CD1d^{-/-}$ mice. The development of liver metastases was evaluated by histopathology. The effect of NK cells on liver metastases was determined by selective depletion with anti-asialo-GM1 antiserum in vivo and NK-cell-mediated cytolysis of B16LS9 melanoma cells in vitro. The role of IL-10 and transforming growth factor (TGF)- β in the inhibition of liver NK resistance to liver metastases was determined in vivo and in vitro neutralization with monoclonal antibodies.

RESULTS. Liver NKT cells, especially type I NKT cells, enhanced liver metastases arising from intraocular melanomas. NKT-cell-deficient mice developed significantly fewer liver metastases that were NK-cell dependent. Tumor-induced liver NKT cells, especially type I NKT cells, inhibited liver NK-cell cytotoxicity by an IL-10-dependent process.

CONCLUSIONS. NKT cells exert protective effects in many murine tumor models. However, the present results reveal that NKT cells exacerbate liver metastases arising from intraocular melanomas. To the authors' knowledge, this is the first report that liver NKT cells, especially type I NKT cells, inhibit liver NK-cell antimetastatic activity by the production of IL-10. These results suggest that hepatic NKT cell activity can have an important effect in the immune surveillance of liver metastases. (*Invest Ophthalmol Vis Sci.* 2011;52:3094-3102) DOI:10.1167/iovs.10-7067

Uveal melanoma is the most common intraocular tumor in adults. Liver metastasis is the leading cause of death in uveal melanoma patients and it has been reported that approximately 95% of patients who die of uveal melanoma have liver metastases.¹ At the present time, there are no therapeutic modalities that significantly control liver metastases or extend

the 5-year survival of patients harboring liver metastases arising from uveal melanomas.² Although immunotherapy has been touted as a promising therapeutic modality, the results to date have been disappointing.^{3,4} A possible explanation is the observation that tumors employ a wide array of strategies for evading immune surveillance. These mechanisms include downregulation of antitumor immune responses by $CD4^{+}CD25^{+}$ regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), M2 macrophages, and natural killer T (NKT) cells.^{3,5,6}

In recent years, it has become clear that innate T cells, such as NKT cells, play an important role in modulating the adaptive immune response.⁷ NKT cells express both T-cell and NK-cell receptors, but unlike conventional T cells that respond to peptides presented by conventional major histocompatibility (MHC) molecules, NKT cells recognize lipid antigens presented by CD1d, a nonclassical MHC molecule. Despite being a small proportion of the total T lymphocyte population (1%–3% of circulating T cells in mice and 0.02%–0.2% in humans),^{8,9} NKT cells are involved in a broad range of immunologic phenomena, including autoimmune diseases, such as type 1 diabetes, graft-versus-host disease, graft rejection, airway hypersensitivity, and cancer.^{7,10,11} CD1d-restricted NKT cells can function as either effector or regulatory cells. In cancer, type I NKT cells exert antitumor effects by producing IFN- γ , which activates NK cells and $CD8^{+}$ T cells and by activating dendritic cells. By contrast, type II NKT cells, which recognize a more diverse array of glycolipids presented by CD1d, inhibit tumor immunity by inducing regulatory cytokines, such as TGF- β , or by recruitment of Tregs.^{11,12} NKT cells also function differently, depending on their anatomic location. Murine liver-derived NKT cells are protective and control tumor growth, unlike thymic and splenic NKT cells, which have far less antitumor effects but have immunoregulatory properties.¹³

The liver is the target organ for metastases arising from uveal melanoma. It also has the highest NKT-cell/T-cell ratio in the body. Up to 50% of the lymphocytes in the liver are NKT cells.^{14–16} Given the wide range of activities mediated by NKT cells, we sought to determine the role that liver NKT cells have in the development of liver metastases arising from intraocular melanomas.

MATERIALS AND METHODS

Cells

The B16LS9 cutaneous murine melanoma cell line was kindly provided by Hans E. Grossniklaus (Emory University School of Medicine, Atlanta, GA). B16LS9 cells were derived from hepatic metastases originating from posterior chamber inoculation of B16-F1 cutaneous melanoma cells in C57BL/6 mice.¹⁷ The cells were maintained in complete DMEM. B16LS9 cells were tested for the expression of CD1d by flow

From the Department of Ophthalmology, University of Texas Southwestern Medical Center, Dallas, Texas.

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Corresponding author: Jerry Y. Niederkorn, Department of Ophthalmology, University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Boulevard, Dallas, TX 75390-9057; jerry.niederkorn@utsouthwestern.edu.

cytometry with anti-CD1d monoclonal antibody (clone 20H2) and were found to be negative (data not shown). Cells were also validated by flow cytometry for H-2^b expression as confirmation of their C57BL/6 origin and tested by ELISA for mycoplasma contamination during the course of this study and were found to be negative (data not shown).

Mice

C57BL/6 mice were purchased from the Wakeland Animal Colony at the University of Texas Southwestern Medical Center (Dallas, TX). Breeding pairs of $\text{J}\alpha 18^{-/-}$ mice (C57BL/6 background), which lack invariant NKT cells, were kindly provided by Joan Stein-Streilein (Schepens Eye Research Institute, Boston, MA), with permission from the originator of the knockout mouse strain (Masaru Taniguchi; RIKEN Research Center for Allergy and Immunology, Yokohama, Japan). These mice have been backcrossed to C57BL/6 mice for nine generations.¹⁸ Breeding pairs of $\text{CD1d}^{-/-}$ mice (C57BL/6 background), which lack both invariant NKT cells and NKT cells expressing diverse T-cell receptors, were kindly provided by Mark Exley (Beth Israel Deaconess Medical Center, Boston, MA) and had been backcrossed to C57BL/6 mice for 12 generations (Exley M, personal communication August 26, 2010). C57BL/6 severe combined immunodeficient (SCID) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All the mice were maintained in a dedicated pathogen-free environment and used between 6 and 14 weeks of age. All the animals were housed and cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the University of Texas Southwestern Medical Center and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Tumor Models and Treatment Approaches

Intraocular tumor inoculation was performed as described previously.¹⁹ This technique produces intraocular melanoma that invades the retina and choroid and causes liver metastasis.^{20,21} B16LS9 tumor cells (1×10^5) were injected into the vitreous cavity (VC). Tumor-bearing eyes were enucleated when the eyes reached 4 mm in diameter. The mice were euthanized 17 days after enucleation or when moribund, and the livers were collected for histology.

Intrasplenic tumor cell injection is an effective method of producing liver metastases, as it facilitates tumor cell dissemination to the liver via the splenic portal-circulatory route.²² Melanoma cells (5×10^4 cells in 100 μL PBS) were injected beneath the spleen capsule, and the mice were euthanized 14 days later or when moribund.

NK cells were depleted by giving 50 μL IP anti-asialo GM1 (asGM1; Wako Pure Chemical, Osaka, Japan) on days -3 , $+1$, and $+5$ relative to tumor cell implantation. To inactivate NKT cells, we injected the mice IP with 50 μg of anti-CD1d (clone 20H2) three times a week, starting on day -7 . In vivo inactivation of CD25^+ Tregs was achieved by IP injection of anti-CD25 (clone PC61 5.3) twice a week beginning 7 days before tumor cell injection.^{23,24} Anti-TGF- β (clone 1D11.16.8) was administered IP (1.0 mg/injection) three times a week starting on day -7 , to neutralize TGF- $\beta 1$ and - $\beta 2$.²⁵ To neutralize IL-10, we treated the mice with 200 μg anti-IL-10 (clone Jes5.2A5), given daily from day -7 through day -1 and once a week thereafter. Cyclophosphamide (Bristol-Myers, Princeton, NJ) was administered IP (100 mg/kg) every 14 days beginning on day -1 .

Splenectomy

The mice underwent splenectomy 7 days before intraocular tumor injection, as described previously.²⁶ Briefly, the mice were anesthetized with ketamine hydrochloride (0.6 mg given IP), and their spleens were exteriorized through an incision in the abdominal wall. The splenic pedicle was severed with scissors. Hemorrhage was arrested by tamponade of the splenic blood vessels. Wounds were closed with stainless-steel wound clips.

Assessment of Liver Metastases

Liver metastases of B16LS9 melanomas were readily demonstrable by histologic examination, as previously described.^{21,27} The livers were sectioned at 100- μm intervals, stained with hematoxylin and eosin (H&E), and examined in a masked fashion by three independent observers. The results were reported as the mean number of metastases per 10 random low-power fields ($\times 100$ magnification).

Liver metastases arising from intrasplenic B16LS9 melanoma injection were assessed by counting surface tumor nodules on the liver with a dissecting microscope.^{28,29} When the liver had more than 200 surface metastatic nodules, the liver metastases were assessed by measuring the area of metastatic tumors from three H&E-stained slides with an ocular micrometer. The results were reported as the mean area of metastasis per 10 random low-power fields ($\times 100$).

In Vitro NK Cell-Mediated Cytotoxicity Assay

Liver mononuclear cells were isolated as described elsewhere²⁹ and were enriched for NK cells with a kit (EasySep Mouse NK Cell Enrichment Kit; Stemcell, Vancouver, BC, Canada). Purified liver NK cells were co-cultured with B16LS9 cells at different E:T ratios for 18 hours, and cytotoxicity was assessed (CytoTox 96 NonRadioactive Cytotoxicity Assay Kit; Promega, Madison, WI). Liver NK cells isolated from Poly I:C-treated mice or anti-asialo GM1-treated mice served as positive and negative controls, respectively.

Flow Cytometric Analysis and Cell Sorting

The number of liver NK cells, MDSCs, and Tregs was assessed by flow cytometry. NK cells were defined as $\text{TCR-}\beta^{-}\text{NK1.1}^+$, or $\text{TCR-}\beta^{-}\text{DX5}^+$ cells. Tregs were identified as $\text{CD4}^+\text{CD25}^+\text{FoxP3}^+$ cells, and MDSCs were identified as $\text{CD11b}^+\text{Gr-1}^+$ cells.

Liver NKT cells were enriched from isolated liver mononuclear cells by cell sorting. A sample of sorted cells was always analyzed for purity. Sorted NKT cell ($\text{NK1.1}^+\text{TCR-}\beta^+$) populations were always $\geq 92\%$ pure. Liver NKT cell production of IL-10 was assessed by treating purified liver NKT cells with Brefeldin A (8 $\mu\text{g}/\text{mL}$) for 90 minutes at 37°C (Sigma-Aldrich, St. Louis, MO). The samples were resuspended in fixation-permeabilization solution containing 1 μg rat IgG_{2b} anti-mouse CD16/CD32 monoclonal antibody (BD Fc Block; BD Biosciences, San Diego, CA) for 20 minutes at 4°C, washed, and resuspended in 50 μL permeabilization-washing buffer (BD Perm/Wash; BD Biosciences). The cells were incubated with either 0.5 μg of anti-IL-10-FITC or isotype control antibody for 30 minutes at 4°C, washed, and resuspended in 250 μL 2% formalin solution. Fluorescence was detected with a flow cytometer (FACScan; BD Biosciences). The results were analyzed with allied software (CellQuest ver. 3.1f; BD Biosciences).

Real-Time RT-PCR

Primers used in real-time RT-PCR that are specific for mouse YM-1 (PPM25130A), mouse Arg-1 (PPM31770A), mouse interleukin-10 (PPM03017B-200), and mouse GAPDH (PPM02946E-200) were purchased from Qiagen (Valencia, CA). Real-time RT-PCR amplifications were performed on an RT-PCR detection system (iCycler IQ; Bio-Rad, Hercules, CA) according to a standard protocol (95°C, 10 minutes and 40 cycles: 95°C, 15 seconds and 60°C, 1 minute).

Statistical Analysis

Results were expressed as the mean \pm SD. Differences between groups were analyzed by Student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

NKT-Cell-Deficient Mice Develop Fewer Liver Metastases Arising from Intraocular or Intrasplenic Melanomas Than Do Mice with Intact NKT Cell Repertoires

To test whether NKT cells play a role in the development of liver metastases arising from intraocular tumors, we injected B16LS9 tumor cells into the VC of C57BL/6 wild-type mice; $J\alpha 18^{-/-}$ mice, which lack type I NKT cells; and $CD1d^{-/-}$ mice, which lack both type I and II NKT cells. Tumor-bearing eyes were enucleated when the eyes reached 4 mm in diameter. The mice were euthanized 17 days after enucleation, and the livers were examined for metastases. The results of three separate experiments demonstrated that the NKT-cell-deficient $J\alpha 18^{-/-}$ and $CD1d^{-/-}$ mice developed significantly fewer liver metastases than did the wild-type mice (Fig. 1A).

Antigens introduced into the eye elicit a systemic downregulation of T-cell immunity termed anterior chamber-associated immune deviation (ACAID),^{30–32} which may have affected immune surveillance of intraocular tumors or their metastases. NKT cells are essential in the development of ACAID.³³ Therefore, NKT-cell-deficient mice would be expected to have elevated T-cell immunity compared with normal mice, which theoretically may develop ACAID in response to the intraocular melanomas. Accordingly, experiments were performed to test whether maneuvers that abrogate ACAID would affect the development of liver metastases. ACAID can be ablated by either splenectomy or low-dose cyclophosphamide treatment before intraocular injection of antigens.³⁴ Accordingly, B16LS9 melanoma cells were injected into the eyes of splenectomized and cyclophosphamide-treated mice, and the development of liver metastases was assessed. The results clearly demonstrate that ablation of ACAID by either splenectomy or cyclophosphamide treatment did not affect the development of metastases (Fig. 1B).

To further rule out the possibility that the unique immunologic properties of the eye contribute to the reduction of liver metastases in NKT-cell-deficient mice, we induced liver metastases via the splenic portal system. Wild-type, $J\alpha 18^{-/-}$, and $CD1d^{-/-}$ mice were injected intrasplenically with B16LS9 tumor cells and assessed for liver metastases 14 days later. The results of these experiments paralleled those found when B16LS9 tumor cells were placed into the eye. That is, the NKT-cell-deficient $J\alpha 18^{-/-}$ and $CD1d^{-/-}$ mice developed significantly reduced liver metastases than did the wild-type mice (Fig. 1C). Thus, we conclude that NKT cells promote the development of liver metastases arising from intraocular melanomas or from blood-borne melanoma cells.

Resistance to Liver Metastases in NKT-Cell-Deficient Mice Is NK-Cell Dependent

Since NK cells play a critical role in the resistance to liver metastases arising from intraocular melanomas in mice and humans,^{32,35} experiments were performed to determine whether the resistance to liver metastases in NKT-cell-deficient mice was NK-cell dependent. Wild-type mice, $CD1d^{-/-}$ mice, and anti- $CD1d$ -treated wild-type mice were depleted of NK cells with anti-asialo GM1 antiserum. The mice were then injected intrasplenically with B16LS9 melanoma cells, and the number of liver metastases was determined 14 days later. NK-cell depletion ablated the resistance to liver metastases in the NKT-cell-deficient mice (Fig. 2A). The microscopic liver metastasis area in the wild-type mice was 45.08 ± 16.3 mm²/30 fields, compared with 61.51 ± 9.62 mm²/30 fields in the anti- $CD1d$ -treated mice or 33.27 ± 11.1 mm²/30 fields in

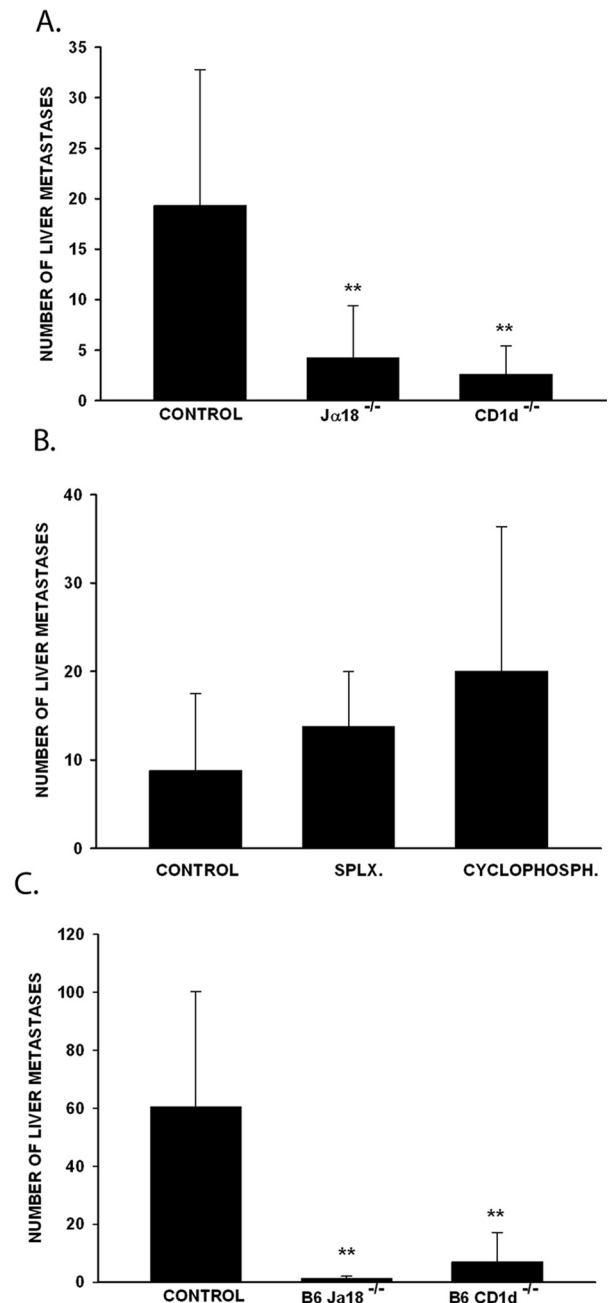
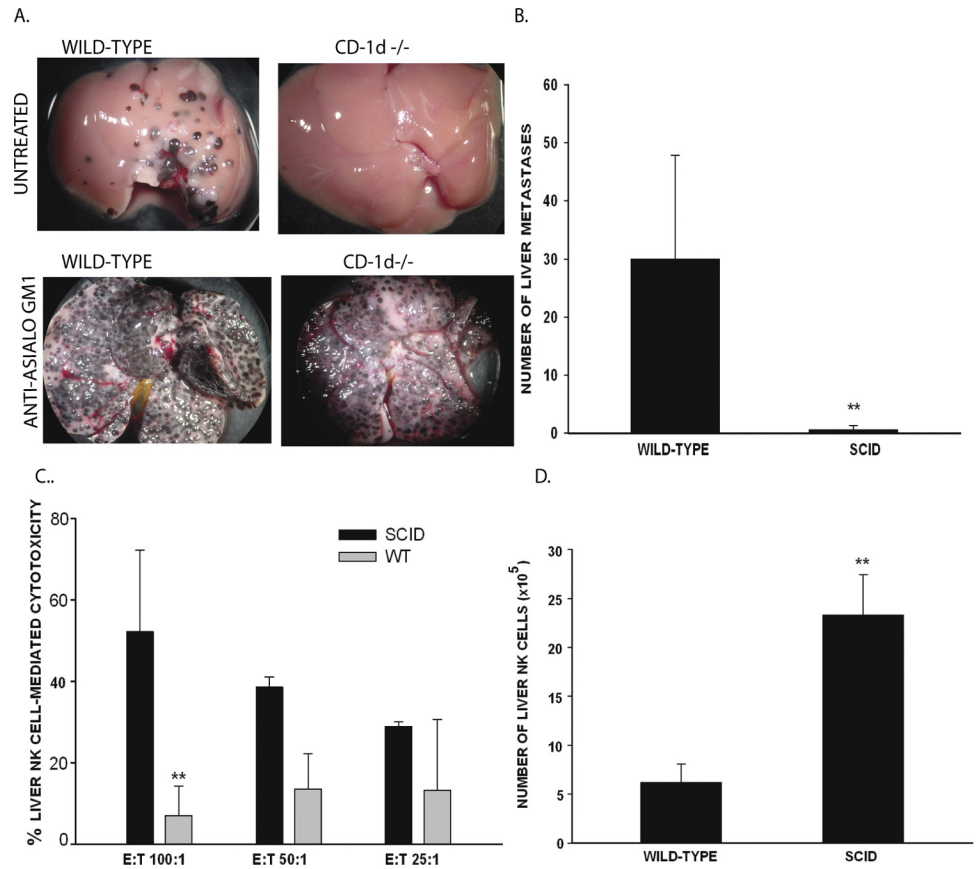


FIGURE 1. NKT-cell-deficient mice developed fewer liver metastases arising from intraocular tumor and intrasplenic tumor injection. (A) Liver metastases in mice harboring intraocular B16LS9 melanomas. (B) Liver metastases in wild-type mice treated by either splenectomy or low-dose cyclophosphamide before injecting melanoma cells into the VC. (C) Liver metastases after intrasplenic injection of B16LS9 melanoma cells. ** $P < 0.01$. All three experiments were performed three times with similar results. $n = 10$ mice/each group. Results are expressed as the mean \pm SD.

$CD1d^{-/-}$ mice. An important finding that the differences in the liver metastasis areas in the NKT-cell-deficient mice and wild-type mice were not significantly different when the hosts' NK-cell repertoire was disabled with anti-asialo GM1 ($P > 0.05$) thus provides further evidence that NK cells play a key role in controlling the liver metastases of B16LS9 tumors. However, NK-cell-dependent resistance to liver metastasis is profoundly downregulated by NKT cells. Although B16 melanomas are notoriously weak immunogens, it was still important

FIGURE 2. Resistance to liver metastases in NKT-cell-deficient mice was NK-cell dependent and T-cell independent. (A) Liver metastases in mice before and after NK cell depletion. This experiment was performed twice with similar results. $n = 5$ mice/group/experiment. (B) Liver metastases in SCID mice and in wild-type mice that received an intrasplenic injection of B16LS9 melanoma cells. Results represent pooled results from three independent experiments. (C) Liver NK cell-mediated cytotoxicity in SCID mice and wild-type mice bearing B16LS9 liver metastases induced by intrasplenic melanoma cell injection. The results are pooled from two independent experiments. (D) Number of liver NK cells (DX5⁺TCR- β ⁻) detected by flow cytometry in mice bearing liver metastases produced by intrasplenic injection of B16LS9 melanoma cells. This experiment was performed twice with similar results ($n = 5$ mice/group/experiment). ** $P < 0.01$. Results are expressed as the mean \pm SD.



to rule out a possible role of T-cell-dependent resistance. Therefore, B16LS9 melanoma cells were injected intrasplenically in SCID mice that are devoid of T cells, but have an intact NK-cell repertoire. The results indicate that T-cell-dependent immunity did not protect against liver metastases; in fact, the SCID mice displayed a remarkable resistance to liver metastases (Fig. 2B). One explanation for the reduced liver metastases in the SCID mice lies in their elevated liver NK-cell activity, which at an E:T ratio of 100:1 was five times higher than that in the wild-type mice (Fig. 2C). Moreover, the SCID mice had a fivefold higher number of liver NK cells compared with the wild-type mice (Fig. 2D).

Melanoma-Induced Liver NKT Cells Inhibit Liver NK-Cell-Mediated Cytotoxicity

Since the remarkable resistance of SCID mice to liver metastases correlated with an increased number of liver NK cells, we considered the hypothesis that a similar condition was present in the NKT-cell-deficient mice and accounted for their resistance to liver metastases. Wild-type and CD1d^{-/-} mice were injected intrasplenically with B16LS9 cells. Liver lymphocytes were isolated at day 14 and the number of liver NK cells was evaluated by flow cytometry. The number of liver NK in the melanoma-bearing CD1d^{-/-} mice was similar to that in the normal and melanoma-bearing wild-type mice (Fig. 3A).

Another explanation for the reduced liver metastases in NKT-cell-deficient mice is that NKT cells in wild-type mice suppress the cytotoxicity of the liver NK cell. This notion was tested by comparing the *in vitro* NK-cell-mediated killing of B16LS9 melanoma cells by liver lymphocytes isolated from non-tumor-bearing naïve wild-type mice, anti-CD1d-treated mice, J α 18^{-/-} mice, and CD1d^{-/-} mice. Liver lymphocytes isolated from each of these groups of naïve mice demonstrated

similar cytotoxicity against B16LS9 melanoma cells, which suggested that normal mice with liver NKT cells do not constitutively express higher NK-cell-mediated cytotoxicity in the liver (Fig. 3B). Additional experiments were performed to test whether the presence of melanoma induces NKT cells to exert a suppressive effect on liver NK cells. The mice were injected intrasplenically with B16LS9 tumor cells, and 14 days later, liver NK cells from different groups of mice were isolated and tested in an *in vitro* NK-induced killing assay. Liver NK cells from J α 18^{-/-}, CD1d^{-/-}, and anti-CD1d-treated mice caused significantly higher cytotoxicity in the B16LS9 cells than in the wild-type mice (Fig. 3C), which indicates that melanoma liver metastases induce liver NKT cells to downregulate NK-cell-mediated cytotoxicity in the liver.

Downregulation of Liver NK Cell Activity by NKT Cells Is IL-10 Dependent but TGF- β Independent

IL-10 and TGF- β are two important cytokines that regulate NK-cell function.³⁶⁻³⁹ Experiments were performed to determine whether the suppression of liver NK cytotoxicity by tumor-induced NKT cells was IL-10 or TGF- β dependent. Wild-type mice, anti-CD1d-treated mice, and J α 18^{-/-} mice were treated with either anti-IL-10 or anti-TGF- β and injected intrasplenically with B16LS9 cells. Liver metastases were assessed as before. Anti-IL-10-treated wild-type mice had a six-fold reduction in liver metastases than did the rat IgG isotype control-treated wild-type mice (Fig. 4A). The wild-type mice treated with anti-IL-10 and the J α 18^{-/-} mice developed a similar number of liver metastases, demonstrating that the enhanced development of liver metastases in NKT-competent mice was IL-10 dependent. These results were confirmed by disabling NKT cells in wild-type mice by administering an anti-CD1d antibody (Fig. 4B). To confirm that the reduced liver NK-cell

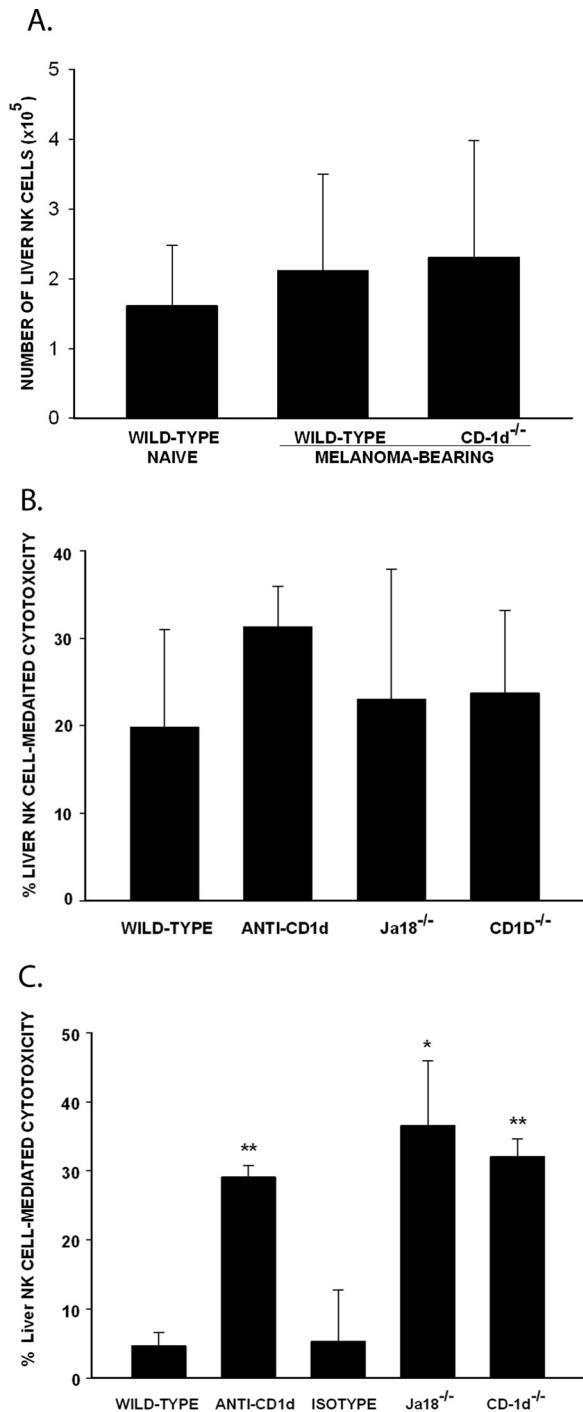


FIGURE 3. Liver metastasis-induced NKT cells inhibited NK-cell-mediated cytotoxicity against B16LS9 melanoma cells. (A) Number of liver NK cells (NK1.1⁺TCR-β⁻) detected by flow cytometry in mice harboring liver metastases induced by intrasplenic injection of B16LS9 melanoma cells ($n = 5$ mice/group/experiment). (B) Cytotoxicity of liver NK cells against B16LS9 tumor cells in naive mice. (C) Liver NK cell cytotoxicity against B16LS9 tumor cells in tumor-bearing mice. All experiments were repeated twice. * $P < 0.05$; ** $P < 0.01$. Results are expressed as the mean \pm SD.

activity in tumor-bearing mice was due to IL-10, wild-type mice were treated with anti-IL-10 and injected intrasplenically with B16LS9 cells. At day 14, liver NK cells were isolated and used as effector cells in an in vitro assay of NK-killed B16LS9 cells. Anti-IL-10 treatment restored liver NK-cell cytotoxicity in the

wild-type mice to a level similar to that in the NKT-deficient Ja18^{-/-} mice (Fig. 4C). However, when the melanoma-bearing wild-type mice were treated with anti-TGF-β or isotype control IgG, they developed a similar number of liver metastases, indicating that TGF-β was not involved in the inhibition of liver NK-cell activity in this model (Fig. 4D).

Tumor-Induced NKT Cells Produce IL-10 to Suppress the Antimetastatic Effects of Liver NK Cells

It is known that IL-10 can be produced by macrophages, dendritic cells (DCs), MDSCs, B-cells, NKT cells, and Tregs.^{40–44} Experiments tested the hypothesis that tumor-induced liver NKT cells recruit Tregs, MDSCs, and M2 macrophages into the liver to produce IL-10, which subsequently suppresses liver NK-cell activity. Livers from naive mice and wild-type mice with liver metastases were assessed for the presence of CD4⁺ Tregs and MDSCs by flow cytometry and for gene expression of FoxP3, YM-1, and arginase-1 (Arg-1) by qPCR. The expression of FoxP3 reflects the functional status of Tregs,⁴⁵ whereas the expression of YM-1 and Arg-1 correlates with M2 macrophages.

The number of liver CD4⁺CD25⁺FoxP3⁺ Tregs, mean fluorescence intensity (MFI) of FoxP3 expression, and the FoxP3 gene expression in liver mononuclear cells were similar in the naive mice, tumor-bearing wild-type mice, and NKT-cell-deficient mice (data not shown; $P > 0.05$). To further rule out the involvement of Treg IL-10 production, mice were treated with anti-CD25 to disable Tregs.^{46,47} The anti-CD25-treated wild-type mice had several liver metastases, similar to the number in the rat IgG isotype controls, which demonstrated that Tregs are not involved in the development of liver metastases (Fig. 5A). The number of MDSCs was also similar in the naive mice, tumor-bearing wild-type mice, and NKT-cell-deficient mice ($P > 0.05$; Fig. 5B). Gene expression of YM-1 or Arg-1 was not significantly different between the tumor-bearing wild-type mice and NKT cell-deficient mice, which indicates that the tumor-bearing wild-type mice and NKT-cell-deficient mice have a similar number of M2 liver macrophages (Figs. 5C, 5D). Thus, Tregs, MDSCs, and M2 macrophages are not the source of IL-10 in the livers of tumor-bearing mice.

NKT cells can also produce immunoregulatory cytokines, such as IL-10, after activation. This phenomenon was examined by isolating NKT cells from the livers of naive mice and wild-type mice bearing liver melanoma metastases and probing for IL-10 gene expression by qPCR and assessing IL-10 protein expression. Liver NKT cells from the melanoma-bearing mice displayed significantly higher levels of IL-10 gene expression (Fig. 6A) and IL-10 protein expression (Fig. 6B) compared with that in the naive mice. By contrast, IL-10 gene expression was similar in non-NKT cells isolated from the livers of the naive mice and those bearing liver melanoma metastases.

DISCUSSION

It is widely believed that NKT cells can influence the immune response to tumors. Type I NKT cells exert antitumor effects, whereas type II NKT cells generally promote malignancy and impair antitumor immunity.¹¹ However, in the present study, type I NKT cells played a role in enhancing the development of liver metastases arising from either intraocular melanomas or via intrasplenic tumor injection. That is, the Ja18 KO mice, which lack type I NKT cells, but have an intact type II NKT cell repertoire, developed significantly fewer liver metastases than did the wild-type mice, which have an intact type I NKT-cell population. If type I NKT cells play a role in restricting the development of liver metastases, one would expect results the

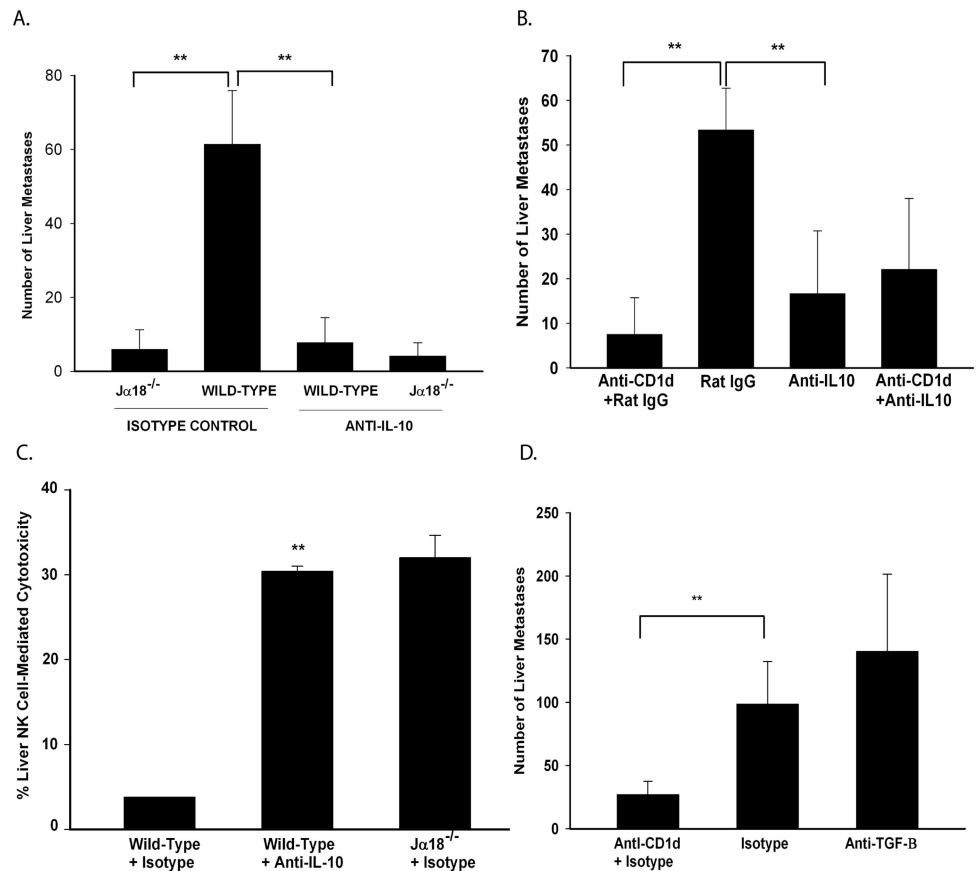


FIGURE 4. Reduced liver metastasis in NKT-cell-deficient mice was IL-10 dependent and TGF- β independent. **(A)** Liver metastases in mice treated with anti-IL-10 or rat IgG isotype. $n = 15$ for all groups. **(B)** Liver metastases in anti-CD1d-treated wild-type mice after anti-IL-10 treatment. $n = 15$ for all groups. **(C)** Liver NK cell cytotoxicity in mice bearing melanoma metastases induced by intrasplenic injection of B16LS9 cells and treated with anti-IL-10 or rat IgG isotype control. The experiment was performed three times with similar results. **(D)** Liver metastases in mice treated with anti-TGF- β or mouse IgG isotype control. $n = 15$ for all groups. The experiment was performed twice with similar results. $**P < 0.01$. Results are expressed as the mean \pm SD.

opposite of those reported here. That is, mice deficient in type I NKT cells would have more, not fewer, liver metastases. On first blush, the reduced number of liver metastases in the $CD1d^{-/-}$ mice, which lack both type I and II NKT cells, suggests that the deficiency in type II NKT cells in these mice accounted for the increased resistance to liver metastases, as type II NKT cells are known to have an inhibitory effect on tumor immunity. However, the resistance to liver metastases was also observed in $J\alpha 18$ KO mice, which lack type I NKT cells, but have an intact type II NKT cell repertoire. Thus, the resistance to liver metastases persisted, even in the presence of type II NKT cells and occurred only when type I NKT cells were absent.

The mechanism whereby type I NKT cells inhibit hepatic NK-cell activity and promote liver metastases remains to be resolved. However, several explanations come to mind. Type I NKT cells can produce IFN- γ , which could inhibit NK activity by (1) upregulating indoleamine dioxygenase (IDO), which is known to inhibit NK activity;⁴⁸; (2) upregulating MHC class I molecules on melanoma cells, which send "off" signals that silence the cytolytic machinery of the NK cells¹³; and (3) disabling the perforin-mediated cytolytic pathway used by the NK cells.⁴⁹

The results also indicate that the enhanced resistance to liver metastasis in mice deficient or depleted of NKT cells was not due to the ablation of ACAID, which requires the presence of an intact NKT cell repertoire.³³ However, the resistance to liver metastasis is NK-cell dependent and T-cell independent. NKT-cell-competent mice and NKT-cell-deficient mice have a similar number of liver NK cells, which express comparable levels of NK-cell-mediated cytotoxicity against B16LS9 melanoma cells. The data indicate that the liver NKT cell repertoire is altered by the presence of melanoma metastases in the liver

and as a result, suppresses NK-cell cytotoxicity through the production of IL-10.

Other studies have shown that NKT cells can have dual roles in tumor immunity by either exerting antitumor effects or by suppressing antitumor immune responses.^{7,10,48} NKT cells derived from different organs also function differently. Those residing in the liver typically express antitumor properties.¹³ However, the present findings indicate that the presence of melanoma liver metastases converts NKT cells to a suppressive phenotype. This curious departure from other tumor models may be related to the origin of the B16LS9 melanoma cell line, which was derived from liver metastases of B16-F1 cells injected into the ocular posterior chamber of C57BL/6 mice.¹⁷ The effect of B16LS9 on the development of liver metastases appears to be organ-specific. That is, there were no differences in the growth of B16LS9 in the VC of the eye or the spleens in the $J\alpha 18$ KO mice, $CD1d$ KO mice, and wild-type C57BL/6 mice. Experiments are in progress to determine whether tumors that successfully colonize the liver undergo epigenetic changes that influence their interactions with NKT cells.

In the present study, we found that the $J\alpha 18^{-/-}$ mice and $CD1d^{-/-}$ mice had fewer liver metastases arising from intraocular B16LS9 melanoma that was not related to the ablation of ACAID, which is a form of systemic immune tolerance that is induced when antigens enter the eye. However, the resistance to liver metastases that emerged when NKT cells were eliminated, either by gene deletion or by anti-CD1d treatment, correlated with elevated liver NK-cell activity. This result is in keeping with those from animal studies and findings in uveal melanoma patients. NK cells are known to play a major role in controlling liver metastases from intraocular melanomas in mice.²¹ Moreover, there is a strong correlation between liver NK-cell activity and resistance to liver metastases in uveal

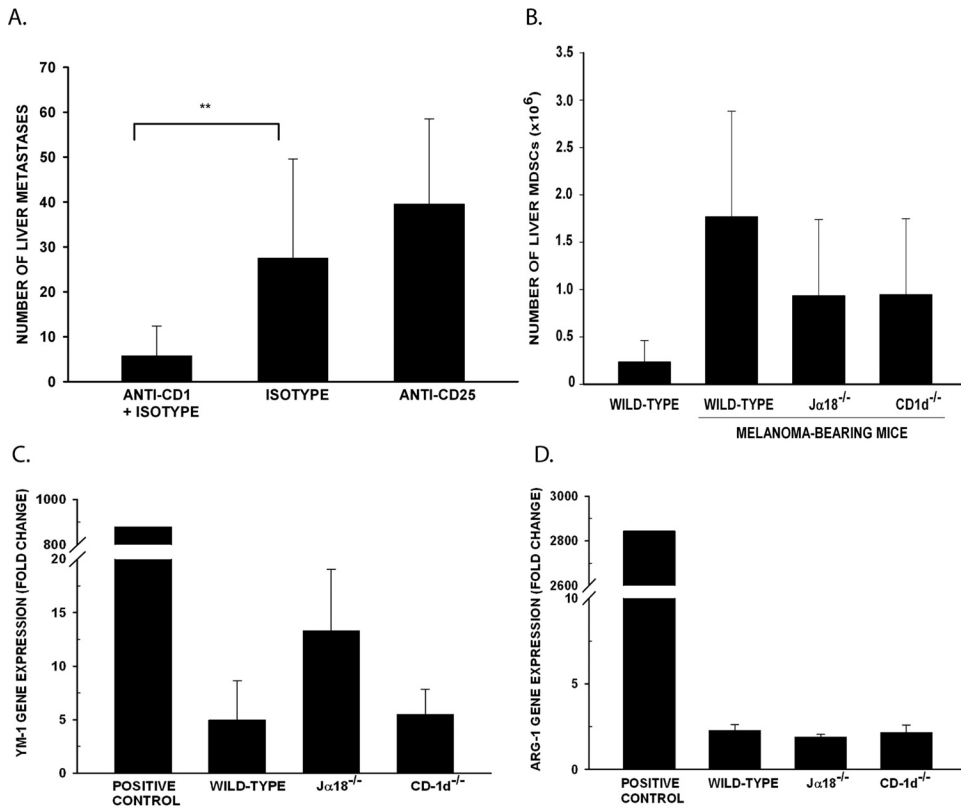


FIGURE 5. Role of Tregs, MDSCs, and M2 macrophages in IL-10 production in liver metastases produced by intrasplenic injection of B16L9 melanoma cells. (A) Liver metastases in mice treated with either anti-CD25 or rat IgG isotype control. *n* = 15 for all groups. (B) Number of liver MDSCs (CD11b⁺Gr-1⁺) detected by flow cytometry. (C) Expression of YM-1 and (D) Arg-1 genes detected by qPCR using liver mononuclear cells of mice bearing melanoma metastases. Raw 264.7 cells treated with IL-4, -10, and -13 served as the positive control. ***P* < 0.01. Results are expressed as the mean ± SD.

melanoma patients.⁴⁹ The results reported here lend further support to the notion that NK cells play a critical role in controlling melanoma metastases in the liver. In particular, our findings indicate that NKT cells can profoundly suppress liver NK-cell activity and create a local environment that favors the emergence of melanoma liver metastases. Liver NKT cells appear to produce their suppression of liver NK cells through the elaboration of IL-10, but not via TGF-β. The reversal of suppressed NK activity and the restoration of resistance to liver metastases that follows *in vivo* treatment with anti-IL-10 antibody further support the hypothesis that liver NKT cells enhance liver metastases by a local suppression of NK cells via IL-10. By contrast, administration of exceptionally high doses of anti-TGF-β antibody (1 mg, three times per week) did not relieve suppressed liver NK-cell activity.

IL-10 can be produced by a wide variety of cells, including those with immunoregulatory properties (e.g., Tregs, MDSCs, M2 macrophages, and NKT cells). The present findings indicate that a deficiency in NKT cells did not affect the number of Tregs, MDSCs, or M2 macrophages in the livers of tumor-bearing mice. IL-10 gene expression in liver non-NKT cells, which included Tregs, MDSCs, and M2 macrophages, from the NKT-cell-deficient mice was the same as that found in the non-NKT cells isolated from the livers of wild-type, tumor-bearing mice, a finding that further suggests that the upregulation of IL-10 was restricted to the liver NKT cells. By contrast, IL-10 gene expression was significantly increased in liver NKT cells isolated from the tumor-bearing mice compared with the naïve mice, which is consistent with the proposition that liver NKT cells from naïve mice do not constitutively suppress liver

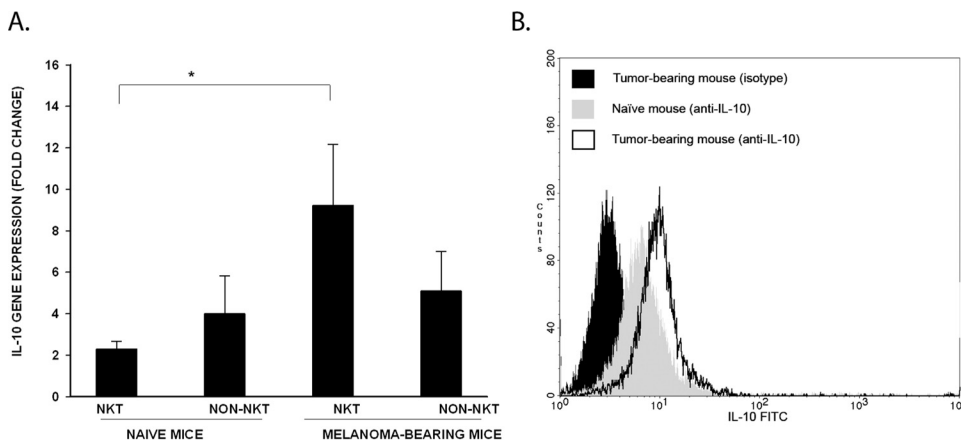


FIGURE 6. IL-10 production by liver NKT cells in mice bearing melanoma liver metastases induced by intrasplenic injection of B16L9 melanoma cells. Liver mononuclear cells were isolated from mice harboring melanoma liver metastases. Mononuclear cells were sorted for NKT cells (NK1.1⁺TCR-β⁺) and non-NKT cells (single-positive cells or double-negative cells). (A) Assessment of IL-10 gene expression by qPCR. **P* < 0.05. Results represent mean ± SD. (B) Production of IL-10 by liver NKT cells. The black-shaded histogram represents liver NKT cells from tumor-bearing mice stained with the isotype control. Liver NKT cells from naive mice stained with the isotype control show similar results (data not shown). The gray-shaded histogram represents IL-10 production by naïve liver NKT cells. The open histogram represents IL-10 production by liver NKT cells from tumor-bearing mice. All results are representative of two independent experiments.

show similar results (data not shown). The gray-shaded histogram represents IL-10 production by naïve liver NKT cells. The open histogram represents IL-10 production by liver NKT cells from tumor-bearing mice. All results are representative of two independent experiments.

NK activity, whereas liver NKT cells from tumor-bearing mice inhibit liver NK-cell activity in an IL-10-dependent fashion. It has been shown that NKT cells can downregulate tumor immune surveillance through the IL-4-STAT 6 pathway, and in a CD8-dependent manner by decreasing CTL activity.^{10,11} To our knowledge, this is the first report that liver NKT cells, especially type I NKT cells, inhibit the antimetastatic activity of liver NK cells via the production of IL-10.

NKT cells, especially type I NKT cells, have been administered to patients with lung cancer, melanoma, glioma, breast cancer, colorectal cancer, liver cancer, kidney cancer, and prostate cancer in several phase I clinical trials.¹⁰ Extrapolating the present finding to human uveal melanoma should be viewed with caution. B16LS9 was derived from the cutaneous B16 murine melanoma and thus, it is not a uveal melanoma. However, some similarities between human uveal melanomas and B16LS9 cutaneous melanomas transplanted into the posterior compartment of the eye bear noting. Human uveal melanomas and B16LS9 melanomas both display a strong predilection to form liver metastases, and both are susceptible to NK-cell-mediated cytotoxicity. It is also important to note that both humans and mice have NKT cells in their livers.^{13,50,51}

A growing body of evidence indicates that type I NKT cells can play both effector and regulatory roles in antitumor immunity. Therefore, it is important to examine the role of NKT cells in different types of tumor models to gain a better understanding of their function. Based on our study, modulation liver NKT cells may be an important consideration when designing immunotherapy in the management of liver metastases in uveal melanoma patients.

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