Prolonged Survival of Human Hepatocarcinoma Cells in the Liver of Newborn C57BL/6 Mice and Resulting Cellular Xenorejection, Especially the Activation of Hepatic Natural Killer T Cells

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Key Words

CD1d • Cellular rejection • Cyclosporin A • Hepatocellular carcinoma • Natural killer T cell • Tumor xenografts

Abstract

Objective: To investigate the fate of human hepatocellular carcinoma (HCC) in the livers of newborn mice and the resulting cellular rejection. **Methods:** Two HCC cell lines (HepG2 and HCCLM3) labeled with DMAHAS were orthotopically transplanted to newborn and adult mice with or without low-dose cyclosporin A (CsA) treatment (10 mg/kg). The fate of tumor xenografts was examined and the resulting cellular response was investigated. **Results:** Tumor xenografts survived in newborn mice for >4 weeks, with a delayed lymphocyte infiltration mediated by CD4⁺ T, CD8⁺ T and NK1.1⁺ cells. In contrast, the xenografts survived in adults <8–10 days with an acute cellular rejection by CD8⁺ T cells, NK1.1⁺ cells, macrophages or neutrophils. Orthotopic transplantation of human HCC xenografts elicited a strong cytotoxic response in newborn mice ($p < 0.05$), and selective T/NK1.1⁺ cell deletion in vitro suggested that such effector cells were mainly CD8⁺ T cells. Moreover, tumor xenografts induced a rapid activation of hepatic natural killer T (NKT)

cells in both newborn and adult mice with enhanced secretion of IL-4 and IFN- γ in serum and subsequent NKT-like cytotoxicity. The rapid activation of NKT cells could be efficiently suppressed by low-dose CsA treatment, possibly in a CD1d-independent manner. **Conclusion:** Our data suggest that the livers of newborn mice were more suitable for the survival of xenografts than those of adult mice. Cell-mediated tumor xenorejection in newborn mice was different from that in adults, and hepatic NKT cells may play an important role in early tumor xenorejection.

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Introduction

Tumor xenograft models are widely used for human tumor biology, chemotherapy and immunotherapy analysis. Recently, many effective immunotherapies have been obtained from tumor xenograft models by injecting tumor-specific monoclonal antibodies, or adoptive transfer of cytotoxic T lymphocytes (CTLs) or natural killer T (NKT) cells [1–4]. Some have been effectively used in cancer patients, with partial success in others [5–7], suggesting that the antitumor immune response occurring

in immunodeficient tumor xenograft models may be different from that in cancer patients. Studies performed to elucidate the immune mechanisms of tumor-host interactions in immunocompetent tumor xenograft models will increase our understanding of tumor xenorejection. Previous studies have demonstrated that tumor xenografts can grow in some immunologically privileged sites, such as the anterior chamber of the eye, the hamster cheek pouch, or under the kidney capsule of mice or rats with limited cyclosporine A (CsA) treatment [8, 9]. The potential barriers to successful xenograft survival are believed to be T-cell-mediated xenorejection and tissue microenvironments. The importance of T cells in xenorejection can be manifested by the growth of xenografts in mutant nude mice. However, the interaction between tumor xenografts and their local microenvironment has received little attention.

As a common site for human hepatocellular carcinoma (HCC) transplants, the liver is accepted as a complex immune or tolerance organ in recent years. It contains abundant innate immune cells, such as NK, NKT, Kupffer and dendritic cells (DCs). Besides, the ratio of classic CD4⁺ to CD8⁺ T cells is reversed in the liver in comparison to that in the peripheral blood [10]. These hepatic lymphocytes are able to lyse target cells upon T cell receptor (TCR)-mediated cytotoxicity, which is similar to circulating T cells [11]. All these innate and adaptive immune cells residing in the liver dominate local tumor surveillance, but their role in xenograft survival has been inadequately explored.

NKT cells are specialized T cells coexpressing T cell and NK cell surface markers (NK1.1 in C57BL/6 mice). They develop in the thymus and migrate to the periphery at least 1 week after birth [12], then mature in the peripheral tissue and acquire NK lineage receptors, including NK1.1 [13]. Most of them are CD1d restricted, which is a non-classical MHC I molecule presenting lipids to NKT cells [13]. Once activated, NKT cells can rapidly secrete IFN- γ and IL-4 [14], which activate downstream NK cells, DCs, B and CD8⁺ T cells [13] to inhibit tumors [15, 16], fight infections [17, 18] and prolong allotransplant survival [19–21]. However, the role of NKT cells in tumor xenograft survival differs in different models [3, 4, 22–24]. The activity of NKT cell subsets may be affected by the type and size of xenografts, the transplantation site and the immune status of recipients. Currently, little is known about hepatic NKT-cell-mediated human tumor rejection in immunocompetent mice.

In the present study, we focus on the immune changes in the liver microenvironment of newborn mice induced

by human HCC xenografts, especially the activation and function of hepatic NKT cells. We have examined the fate of two different lines of HCC cells in newborn and adult mice, as well as the resulting cellular rejection. The data demonstrated that the livers of newborn mice were more suitable for the survival of human HCC xenografts than those of immunocompetent adults, and the cell-mediated xenorejection in newborn mice was different from that in adults. Moreover, transplanted tumor xenografts induced rapid activation of hepatic NKT cells in newborn and adult mice, which could be efficiently suppressed by low-dose CsA treatment.

Materials and Methods

Mice

C57BL/6 (B6) mice were purchased from the Experimental Animal Center of the Shandong University. They were maintained under pathogen-free conditions, and given standard food and sterilized water. Mice were used at two developmental stages: 3 days after birth (newborn, $n = 96$) and at 6–8 weeks of age (adult, $n = 48$). All procedures were conducted in accordance with the protocol approved by the Institutional Animal Care and Use Committee.

Cell Lines

Two α -fetoprotein (AFP)-positive human HCC cell lines were used in this study. The HepG2 cell line was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and the HCCLM3 cell line was purchased from the Liver Cancer Institute and Zhongshan Hospital of Fudan University (Shanghai, China). HCCLM3 cells have hepatitis B virus DNA integrated in their genome, and exhibit high metastatic potential in nude mice [25]. The cells were maintained in high-glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and were cultured in a humidified incubator with 5% CO₂ at 37°C.

DMAHAS Labeling

DMAHAS [*Trans*-4-(N-2-hydroxyethyl-N-ethyl amino)-4'-(dimethyl amino) stilbene], a new dye with green grain crystals, was synthesized by the State Key Laboratory of Crystal Materials, Institute of Crystal Materials, Shandong University (Jinan, China). DMAHAS exhibits strong one- or two-photon-induced blue fluorescence at 436 nm [26]. In vitro cytotoxicity evaluation showed that DMAHAS had no significant toxicity on HepG2 or HCCLM3 cells, and our in vivo experiments demonstrated that DMAHAS disappeared in the recipient site after labeled cells underwent apoptosis or necrosis and were subsequently cleared by the host, suggesting that it was a reliable probe for tumor cell imaging [27]. DMAHAS was dissolved in ethanol at a stock concentration of 0.1 M and stored at 4°C. Cultured cells were incubated at a final concentration of 10 μ M at 37°C for 30 min. The cells were then collected and washed with phosphate-buffered saline three times to remove the residual dye. The labeled cells were suspended in basic medium and adjusted to 1×10^8 cells/ml. Cell viability was >95%, as assessed by trypan blue exclusion.

Orthotopic Implantation of Human HCC Cells in Immunocompetent Newborn and Adult Mice

The 3-day-old (newborn, $n = 96$) and 6- to 8-week-old (adult, $n = 48$) B6 mice were used as recipients for the orthotopic implantation of human HCC cells. They were randomly divided into four treatment groups: a HCCLM3 intrahepatic injection group, a HepG2 intrahepatic injection group, a HCCLM3 intrahepatic injection with low-dose CsA group, and a PBS control group. Tumor cell suspension (2×10^6 cells/20 μ l) was injected into the livers of recipient mice with a 27-gauge glass syringe (Hamilton Bonaduz, Bonaduz, Switzerland). For cell tracing, tumor cells were labeled with DMAHAS as described before. The 3-day-old mice received lidocaine as a local anesthetic, while the adult mice were anesthetized with pentobarbital. The needle was injected at a 30-degree angle into the capsule of the left-lateral hepatic lobe at a depth of 3–5 mm below the skin surface. The needle was then carefully removed and a small piece of sterile gauze was placed on the injection site to reduce bleeding.

CsA Treatment and Serum CsA Detection

CsA was purchased from Sandoz Pharmaceuticals as a liquid suspension (100 mg/ml) and diluted in a physiological salt solution prior to the injection. Low-dose CsA (10 mg/kg of body weight) was injected subcutaneously on a daily basis starting 1 day before tumor implantation. The serum levels of CsA in the treated mice were assessed by fluorescence polarization immunoassay (TDX/FLX, Abbott, Abbott Park, Ill., USA) using a cyclosporine monoclonal-antibody (mAb), whole-blood detection kit (49184Q100). The average level of CsA detected was 125 ng/ml ($n = 5$).

Pathological Examination of Xenografts

To examine the outcome and histological features of the transplanted human tumors in the recipient mice, both tumor xenografts and adjacent normal tissue were removed at given time points. Samples were divided and either embedded in OCT for analysis of frozen sections using a fluorescence microscope or fixed in 4% paraformaldehyde for hematoxylin-eosin staining and microscopic examination.

Flow Cytometry

For phenotypic analysis of hepatic and splenic NK/NKT cells, CD4+ T cells and CD8+ T cells, FITC-, PE- or PerCP/Cy5.5-conjugated mAbs against TCR α/β (H57-597), CD3, CD8, NK1.1 (PK136), CD4 and CD1d were purchased from BD Pharmingen (Hamburg, Germany). The annexin V/propidium iodide (PI) apoptosis detection kit was purchased from Beijing Biosea Biotechnology (Beijing, China). Splenic or hepatic lymphocytes were isolated as previously described [28] and stained using mAbs at concentrations of 0.2 μ g/ 10^6 cells. FCM analysis was performed on a FACSCalibur (Becton Dickinson, Biosciences, San Jose, Calif., USA).

NKT-Like or Lymphocytotoxic Activity Analysis

Splenic or hepatic lymphocytes were isolated from tumor-bearing newborn mice on day 16 after implantation, adjusted to a concentration of 1×10^6 /ml, transferred to 96-well plates, and examined for direct cytotoxicity against human HCCLM3 or B6 thymocyte target cells (at a ratio of 25:1, 1×10^4 target cells/well) in a LDH release assay according to the manufacturer's instructions

to analyze the lymphocytotoxic or NKT-like activity. In some experiments, the effector cells from the splenic lymphocytes of tumor-bearing newborn mice were depleted with anti-CD3 (KT3), anti-CD8 (1803), anti-CD4 (H129.19) or anti-NK1.1 (PK136; 10 μ g/ml), as described before [29]. The remaining cells were examined for cytotoxicity against human HCCLM3 cells in a LDH release assay. Assays were performed in triplicate. Percent specific release was calculated as follows: (experimental release – spontaneous release)/(maximum release – spontaneous release) $\times 100$.

IL-4 and IFN- γ ELISA

Sera were collected from the retro-orbital veins. All samples were stored at -80°C . Levels of IL-4 and IFN- γ were measured by ELISA using a quantitative kit (Jingmei, Shenzhen, China), according to the manufacturer's protocols.

Statistical Analysis

The data obtained were analyzed using Student's *t* test. A value of $p < 0.05$ was considered significant.

Results

Pathological Examination of Tumor Xenografts

Apart from 3 newborn mice dying from the implantation procedure, none died from the xenograft burden during the experiment. In newborn mice, the tumor xenografts generated white nodules with well-defined limits in the liver lobe. The tumor masses were often <0.5 cm in diameter and with little regularity in their growth pattern. Based on visual inspection, HCCLM3 and HepG2 xenografts did not differ with respect to survival (table 1). In vitro fluorescence imaging of tumor xenografts showed that both HCCLM3 and HepG2 cells could survive >4 weeks in the livers of newborn mice (fig. 1, HCCLM3 xenografts not shown). Lymphocyte infiltration appearing in and around the xenografts was observed in 8 of 10 (80%) tumor transplants 8 days after implantation. Neither macrophages nor neutrophils were visible, and lymphocytes were recruited in the normal liver tissue near the tumor xenografts (fig. 2a, b). Following the immunological attack from host, the xenografts underwent apoptosis or necrosis (fig. 1g, h, fig. 2c), but 5 of 8 (62.5%) tumor xenografts still survived >4 weeks (table 1), and no evidence of distant metastasis was observed.

In contrast to the prolonged survival of xenografts in newborn mice, xenografts in the livers of adult mice encountered acute cellular rejection by many macrophages or neutrophils on day 2 after transplantation (fig. 2g, h), and they were not found in the livers on day 8 after transplantation (table 1).

Fig. 1. Fluorescent features of the tumor xenograft after implantation in the livers of newborn mice. Tumor xenografts and adjacent normal tissue were sampled 3 (**a, b**), 8 (**c, d**), 21 (**e, f**) and 28 (**g, h**) days after transplantation. Samples for frozen sections were em-

bedded in OCT and analyzed with a fluorescence microscope (**b, d, f, h**). **a, c, e, g** Corresponding images with a light microscope. Original magnification: $\times 200$.

Table 1. Survival of transplanted tumors in newborn and adult mice with or without CsA treatment

Group	Recipient mice	Tumor cells	Survival, days (survived/injected)	Survival rate
1	newborn (n = 26)	HepG2	3(3/3), 8(4/5), 14(3/4), 21(3/5), 28(2/4), 35(1/3), 42(0/2)	61.1% (16/26)
2	newborn (CsA; n = 22)	HepG2	3(2/2), 8(4/5), 14(2/3), 21(3/4), 28(3/4), 35(1/2), 42(1/2)	72.7% (16/22)
3	newborn (n = 23)	HCCLM3	3(3/3), 7(3/4), 14(2/4), 21(3/4), 28(2/4), 35(1/2), 42(0/2)	60.9% (14/23)
4	newborn (CsA; n = 25)	HCCLM3	3(2/2), 7(3/4), 14(4/5), 21(2/4), 28(2/4), 35(3/4), 42(1/2)	68.0% (17/25)
5	adult (n = 12)	HCCLM3	2(2/2), 4(2/3), 8(0/3), 10(0/2), 12(0/2)	33.3% (4/12)
6	adult (CsA; n = 12)	HCCLM3	2(2/3), 4(1/2), 8(0/3), 10(0/2), 12(0/2)	25.0% (3/12)

Hepatic NKT Cell Activation in Newborn and Adult Mice Induced by HCCLM3 Xenografts

In control newborn mice, NKT cells first appeared in the liver and spleen 8 days after birth (fig. 3a). The numbers of NKT cells gradually increased and reached normal levels 5–6 weeks after birth (fig. 4a). After transplanting HCCLM3 cells into the livers of 3-day-old mice, which had immature immunity (fig. 3b), activation of hepatic NKT cells with the down-regulation of NK1.1 expression appeared on days 5 and 10 after implantation (1.00 and 1.02%, vs. 3.25 and 3.16% in the control group, respectively), and NK1.1 molecules reappeared on day 16 after implantation (fig. 3a, fig. 5). No significant down-regulation of NK1.1 expression on the surface of splenic NKT cells was detected (fig. 3a). The absolute number

and proportion of hepatic CD4+ T cells was significantly increased on day 16 after implantation ($p < 0.01$, fig. 3b), whereas the absolute number of NK cells and CD8+ T cells increased in the liver but decreased in the spleen ($p < 0.05$, fig. 3b), though the number of NK cells was elevated in the spleen (fig. 3a).

In adult mice, the proportion of CD8+ cells increased in the liver, but decreased in the spleen 2 days after transplantation (fig. 4b). Interestingly, activation of hepatic NKT cells with loss of NK1.1 surface expression appeared on days 2 and 4 after implantation. Then the NK1.1 marker was reexpressed and returned to normal levels on day 10 after transplantation (fig. 4a, fig. 5). There was no significant change in NKT activation in the spleen after tumor transplantation (fig. 4a).

Fig. 2. Histopathological examination of tumor xenografts (HCCLM3) in newborn and adult C57BL/6 mice. Xenografts and normal adjacent tissue were taken from newborn mice on days 8, 14, 21 and 35 (**a–f**) and on days 2 and 4 from adult mice (**g–i**). All samples were fixed in 4% paraformaldehyde for standard HE staining and microscopic examination (original magnification:

a, g $\times 100$; **b–f, h, i** $\times 200$). **a, b** Day 8 after transplantation (arrows show lymphocyte infiltration). **c** Day 21 after transplantation. **d–f** Low-dose CsA treatment group (10 mg/kg) 8 (**d**), 21 (**e**) and 35 (**f**) days after transplantation. **g–i** Day 2 (**g**: arrows show inflammatory infiltration; **h**) and day 4 (**i**) after transplantation.

Cell Apoptosis of Lymphocytes from the Liver and Spleen of Tumor-Bearing Mice

Hepatic or splenic lymphocytes were isolated from newborn and adult mice 10 and 2 days after tumor xenograft implantation, respectively, and analyzed by flow cytometry (FCM) after annexin V/PI double staining. No significant apoptosis of hepatic or splenic lymphocytes was detected at any given time (fig. 6, data of adults not shown), suggesting that the disappearance of NK1.1+ T cells in tumor-bearing livers was not caused by activation-induced cell apoptosis.

NKT-Like and Lymphocytotoxic Activities Induced by HCCLM3 Xenografts

Hepatic lymphocytes from tumor-bearing newborn mice on day 16 after implantation showed higher NKT-like cytotoxicity against B6 thymocytes at the ratio of 25:1, which could be efficiently suppressed by low-dose CsA treatment ($p < 0.05$; fig. 7b). Meanwhile, lymphocytes isolated from the spleen and liver of tumor-bearing newborn mice showed similar levels of cytotoxicity against HCCLM3 cells 16 days after implantation, which was also suppressed by low-dose CsA treatment ($p < 0.05$; fig. 7a,

cytotoxicity of hepatic lymphocytes not shown). Similar results can be found in adult mice 4 days after implantation (data not shown). In vitro deletion of CD3+ or CD8+ cells efficiently reduced cytotoxicity against HCCLM3 cells ($p < 0.05$). However, in vitro deletion of CD4+ or NK1.1+ cells showed no evident effects (fig. 7c).

CD1d Expression on Total Lymphocytes, CD3+ T Cells and NK1.1+ Cells from the Liver and Spleen of Mice Bearing HCCLM3 Cells

In newborn mice, CD1d expression first increased on hepatic NK1.1+ cells 10 days after transplantation. Up-regulation of CD1d expression was detected on hepatic CD3+ T cells and NK1.1+ cells 16 and 28 days after transplantation. No significant up-regulation of CD1d expression was found on splenic lymphocytes, CD3+ T or NK1.1+ cells from recipient mice (fig. 8a).

In adults, the expression of CD1d first increased in hepatic and splenic lymphocytes, CD3+ T cells and NK1.1+ cells 4 days after implantation. Subsequently, it significantly increased on day 10 even though no xenografts

were found in the livers at that time (fig. 8b). CD1d expression returned to normal 16 days after transplantation (data not shown).

Secretions of IL-4 and IFN- γ in Tumor-Bearing Newborn and Adult Mice

Sera were collected from the retro-orbital veins on days 10 and 16 in newborn mice and on days 4 and 10 in adult mice after transplantation of human HCCLM3 cells. Levels of IL-4 and IFN- γ were measured by ELISA. The tumor xenografts promoted production of IL-4 on day 10 in newborn mice and on day 4 in adult mice ($p < 0.05$). They promoted production of IFN- γ at any given time in newborn and adult mice ($p < 0.05$) compared with control groups (fig. 9).

Effects of Low-Dose CsA Treatment on the Survival of Xenografts and the Activation of NKT Cells

Low-dose CsA treatment significantly reduced lymphocyte infiltration and prolonged the survival of tumor xenografts in newborn mice up to 5 weeks after implan-

Fig. 3. Changes in NK/NKT cells and CD4+ T cells in the liver and spleen of newborn mice with or without CsA treatment. Hepatic and splenic lymphocytes were isolated on days 5, 10 and 16 after implantation. Cells were stained with FITC-conjugated anti-TCR α/β mAb and PE-conjugated anti-NK1.1 mAb, or PE-conju-

gated anti-CD8 mAb and PerCP/Cy5.5-conjugated anti-CD4 mAb. Data were obtained after gating on lymphoid cells. Numbers in quadrants represent percentages of the cell population. **a** Percentages of NK and NKT cells.

Fig. 3. Changes in NK/NKT cells and CD4+ T cells in the liver and spleen of newborn mice with or without CsA treatment. Hepatic and splenic lymphocytes were isolated on days 5, 10 and 16 after implantation. Cells were stained with FITC-conjugated anti-TCR α/β mAb and PE-conjugated anti-NK1.1 mAb, or PE-conju-

gated anti-CD8 mAb and PerCP/Cy5.5-conjugated anti-CD4 mAb. Data were obtained after gating on lymphoid cells. **b** Absolute numbers of hepatic and splenic NKT, NK, CD4+ and CD8+ T cells. Student's t test (for each group, n = 5, * p < 0.05, ** p < 0.01).

Fig. 4. Changes in NK/NKT cells and CD8+ T cells in the liver and spleen of adult mice with or without CsA treatment. Hepatic and splenic lymphocytes were isolated on days 2, 4 and 10 after implantation. Cells were stained with FITC-conjugated anti-TCR $\alpha\beta$ mAb, and PE-conjugated anti-NK1.1 mAb, or PE-conju-

gated anti-CD8 mAb and PerCP/Cy5.5-conjugated anti-CD4 mAb. Data were obtained after gating on lymphoid cells. Numbers in quadrants represented percentages of the cell population. Percentages of NK and NKT cells (**a**) and CD8+ T cells (**b**) are shown.

tation (fig. 2d–f, table 1). Transient tumor cell proliferation appeared 8 days after implantation, causing the enlarged tumor volume without visible leukocyte infiltration (fig. 2d). Tumor cells were gradually eliminated until they were no longer observed in the livers 6–7 weeks after implantation. However, CsA had no obvious effect on the

survival of xenografts in adults (table 1). Besides the inhibiting effects on CD4+ and CD8+ T cells seen in data from newborn and adult mice (fig. 3, fig. 4b), low-dose CsA treatment efficiently suppressed activation of hepatic NKT cells 2 days after implantation in adult mice, without loss of NK1.1 expression in NKT cells (fig. 4a).

Fig. 5. Changes in hepatic NKT cells with loss of surface NK1.1 expression in newborn (a) and adult mice (b) after tumor xenograft transplantation. Hepatic lymphocytes were isolated on days 5, 10 and 16 in newborn (a) and on days 2, 4 and 10 after transplantation in adult mice (b). Cells were stained with FITC-conju-

gated anti-TCR α/β mAb and PE-conjugated anti-NK1.1 mAb and analyzed by FCM. Data were obtained after gating on lymphoid cells. Student's t test (for each group, $n = 5$, * $p < 0.05$, ** $p < 0.01$).

Fig. 6. Analysis of cell apoptosis of hepatic and splenic lymphocytes from newborn mice after orthotopic implantation with human HCCLM3 cells. Hepatic and splenic lymphocytes were isolated 10 days after implantation. Cell apoptosis was analyzed by FCM after annexin V/PI double staining. Student's t test (for each group, $n = 5$, * $p < 0.05$).

Moreover, low-dose CsA treatment inhibited IL-4 and IFN- γ secretion in recipient mice ($p < 0.05$; fig. 9). CD1d expression was not significantly down-regulated in either hepatic or splenic T cells and NK1.1+ cells in mice receiving low-dose CsA treatment (fig. 8).

Discussion

It has been demonstrated that newborn mice are more susceptible to viral oncogenesis than adults due to their immunological immaturity [30]. Besides the immaturity

of the immune system, AFP expression is increased in neonatal liver [31]. AFP functions as a suppressant of the immune system and may help hepatocarcinoma cells escape from immune surveillance [32, 33]. Therefore, we presumed that the liver microenvironment of newborn mice may be more suitable for human tumor xenograft survival than that of adults. Our data confirmed this assumption. Although the two human HCC lines used in the present study had different tumorigenicity, HepG2 cells were found to be non-tumorigenic in immunodeficient mice, whereas HCCLM3 cells could produce extensive metastasis in nude mice by subcutaneous or ortho-

Fig. 7. Lymphocytotoxic and NKT-like activity of tumor-bearing newborn mice was analyzed by LDH release assay. **a** Splenic lymphocytes were isolated 16 days after implantation. Splenic lymphocyte suspension (1×10^6 /ml) was transferred to 96-well plates and examined for direct cytotoxicity against human HCCLM3 target cells (at the following ratios: 5:1, 10:1, 20:1 and 50:1, 1×10^4 target cells/well). **b** Hepatic lymphocyte suspension (1×10^6 /ml) was transferred to 96-well plates and examined for NKT-like cytotoxicity against B6 thymocyte target cells (at a 25:1 ratio, 1×10^4 target cells/well). **c** Splenic lymphocyte suspension (1×10^6 /ml) was transferred to 96-well plates and examined for direct cytotoxicity against human HCCLM3 target cells (at a 25:1 ratio, 1×10^4 target cells/well). In some experiments, the effector cells were depleted with anti-CD3 (KT3), anti-CD8 (1803), anti-CD4 (H129.19) or anti-NK1.1 (PK136; 10 μ g/ml) mAbs. All assays were performed in triplicate, and statistical significance was calculated by Student's t test (* $p < 0.05$).

topic implantation [25]. Based on histological observation, they showed similar survival and induced similar changes in intrahepatic immune responses. Tumor xenografts in newborn mice maintained their in vitro tumor characteristics with normal expression of AFP and survivin [34]. However, tumor xenografts transplanted into the adult liver induced acute cell rejection shortly after transplantation with transient survival. All this supported the possibility that the liver microenvironment in neonates is more favorable to the survival of HCC xenografts than that in adults.

Next we tried to study what caused such difference in survival of tumor xenografts in newborn and adult mice, and how the cell-mediated xenorejection occurred in their liver and spleen. We found that tumor xenografts induced different patterns of cell-mediated rejection in newborn and adult mice. In newborn mice, transplanted tumors resulted in delayed cell rejection mediated mainly by CD4+ T cells, CD8+ T cells and NK1.1+ cells. However, in adults, the acute cellular xenorejection induced by tumor

xenografts was mediated mainly by CD8+ T cells, NK1.1+ cells, and a large number of macrophages or neutrophils. The different patterns of cell-mediated rejection occurring in newborn and adult mice may be due to the immaturity of immune cells, in particular T cells. As mentioned above, a very low percentage of CD4+ or CD8+ T cells appeared in the liver and spleen of newborn mice 1 week after birth. Though NK cells in the newborn liver had high cytotoxicity, as reported by Bai et al. [35], their percentage was comparable with that in adult liver. Without enough mature CD4+ or CD8+ T cells involved, NK cells as well as the macrophages were unable to reject the tumor xenografts in newborn mice. This has been demonstrated by previous studies in adult mice in which CD4+ T-cell-activated macrophages are required for cell xenograft rejection [36, 37]. NK cells and macrophages fail to reject xenografts without T-cell-mediated activation and recruitment in severe combined immunodeficiency or Rag-/- mice with normal NK cell and macrophage components [38, 39]. With transplant time prolonged, the im-

Fig. 8. Changes in CD1d expression on splenic and hepatic lymphocytes, T cells and NK1.1+ cells from tumor-bearing newborn and adult mice. Hepatic and splenic lymphocytes were isolated and stained with FITC-conjugated anti-TCR α/β mAb, PE-conju-

gated anti-NK1.1 mAb and PerCP/Cy5.5-conjugated anti-CD1d mAb. Data were obtained after gating on lymphoid cells. **a** CD1d expression in newborn mice. **b** CD1d expression in adult mice. --- = Isotype control; — = CD1d.

Fig. 9. Levels of IL-4 (a, c) and IFN- γ (b, d) in the serum of newborn (a, b) and adult mice (c, d) with or without CsA treatment. Sera were collected from the retro-orbital veins on days 10 and 16 in newborn mice and on days 4 and 10 in adult mice. Levels of IL-4 and IFN- γ were measured by ELISA using a quantitative kit. Student's t test (for each group, $n = 3$, * $p < 0.05$; CsA treatment groups were compared with HCCLM3 implantation groups).

immune system of newborn mice matured, and increased numbers of CD4⁺/CD8⁺ T cells or NKT cells migrated into the liver and spleen, evoking an immune attack on tumor xenografts and finally leading to eradication by the host 6 weeks after transplantation. Therefore, splenic or hepatic lymphocytes isolated from newborn mice on day 16 and adult mice on day 4 after transplantation showed similar levels of cytotoxicity on tumor cells, suggesting that even with immature immunity, newborn mice can generate strong cell immunity under appropriate xenoantigen stimulation resembling that of adults [29]. In vitro deletion of CD3⁺, CD4⁺ and CD8⁺ T cells or NK1.1⁺ cells indicated that such cytotoxic effectors were mainly CD8⁺ T cells. Little is known about the cellular xenorejection occurring in immature hosts. We cannot adequately explain how different patterns of cellular xenorejection have occurred in newborn and adult mice.

Interestingly, HCCLM3 xenografts induced rapid activation of hepatic NKT cells with loss of NK1.1 expression both in newborn mice on day 5 and in adult mice 2 days after transplantation. No significant apoptosis of hepatic or splenic lymphocytes was detected, suggesting that such disappearance of NK1.1⁺ T cells in tumor-bearing livers was not caused by activation-induced cell apoptosis. In vivo NKT activation with loss of NK1.1 ex-

pression can be found in α -GalCer-induced antitumor activity and bacterial infections in mice [13, 18]. Activated NKT cells can secrete a large amount of cytokines, in particular IL-4 and IFN- γ , which activate T cells, B cells, NK cells and DCs to participate in many immune responses [13]. In most cases, activation of NKT cells is CD1d restricted, and NKT cells cannot develop in CD1d-deficient mice [40]. Currently, the intriguing role of NKT cells in the inhibition of tumor growth and development attracts attention, and antitumor activity of NKT cells is believed to act via perforin- or FasL-mediated cytotoxicity [41, 42]. It has been demonstrated that adoptive transfer of NKT cells could efficiently suppress the growth and metastasis of Hep3B cells in athymic BALB/c mice [3, 4]. Moreover, the NKT cell ligand α -GalCer can induce the antitumor activity of NKT in a CD1d-dependent manner, and α -GalCer or α -GalCer-pulsed DCs have been used in phase I clinical trials in patients with advanced cancer [43, 44]. Several studies using FCM analysis of hepatic or circulating NKT cells supported that NKT cells have antitumor activity in cancer patients [45–47]. However, one immunohistochemical study of hepatic NKT cells in patients with HCC suggested that NKT cell numbers are not affected by the occurrence of HCC [48]. In the present study, we detected elevated production of IL-4 and IFN- γ

in the serum and higher NKT-like cytotoxicity in the liver after NKT activation induced by tumor xenografts, and increased CD1d expression in T cells and NK1.1+ cells in both tumor-bearing newborn and adult mice, implying that hepatic NKT cells participated in early cell-mediated xenorejection and CD1d molecules may take part in NKT activation. At present, the nature of tumor-derived ligands for NKT cells remains unclear. One report has shown that purified phospholipids extracted from tumors weakly stimulate some NKT cells when loaded on CD1d [49]. Therefore, the detailed mechanism of the interaction between NKT cells and tumor xenografts needs further study.

Low-dose CsA treatment efficiently suppressed activation of hepatic NKT cells, subsequent IL-4 and IFN- γ secretion, and NKT-like cytotoxicity induced by HCCLM3 xenografts, although it had little effect on the survival of xenografts in adult compared with newborn mice. Furthermore, low-dose CsA treatment did not down-regulate CD1d expression in hepatic and splenic lymphocytes of recipient mice, implying that this suppression may occur in a CD1d-independent manner. At present, CsA is widely used in clinical organ transplantation and animal tumor models for its well-known inhibiting effects on T cells. However, little is known about the effects of CsA on NKT cell function. A recent study showed that CsA can suppress NKT cell activation induced by α -GalCer both in the liver and spleen [37].

Until now, it remains difficult to fully elucidate the cellular rejection and the *in vivo* activation of NKT cells induced by tumor xenografts in immunocompetent hosts. Our study only shows one aspect of cellular xenorejection and NKT activation in immunocompetent mice induced by human tumor cells. Moreover, the liver is a complex immune organ and contains many immune cells other than lymphocytes, such as Kupffer cells and DCs, which also express CD1d. They may participate in xenotumor rejection and NKT cell activation. Therefore, further studies exploring the role of NKT cells in cellular xenorejection are needed.

In conclusion, this study provides new information on tumor xenotransplantation in newborn hosts and offers new insights into NKT-mediated cell responses in tumor xenorejection. Moreover, it adds to our understanding of the immunosuppressive effects of CsA.

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