Int. J. Cancer: **115,** 443–449 (2005)

Suppression of hepatocellular carcinoma by transplantation of *ex-vivo* immune-modulated NKT lymphocytes

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NKT cells are a regulatory subset of T lymphocytes with immune modulatory effects and an important role in anti-tumor immunity. The feasibility of "*ex-vivo* education" of NKT cells has recently been demonstrated. To evaluate the anti-tumor effect of *ex-vivo* immune-modulated NKT lymphocytes in a murine model of hepatocellular carcinoma. Athymic Balb/C mice were sublethally irradiated and transplanted with human Hep3B HCC. NKT cells prepared from immunocompetent Balb/C mice were pulsed *ex vivo* with HCC-derived antigens (Group A), Hep3B cells (group B) or BSA (group C), and adoptively transferred into HCC harboring mice (1×10^6 NKT cells per mouse). Group D mice did not undergo NKT cell transplantation. Group E mice were transplanted with 1×10^6 NKT cells from HBV-immunized donors. Mice were followed for tumor size and weight. To determine the mechanism of the anti-tumor effect, intrasplenic lymphocyte populations were analyzed by FACS for NKT, CD4+ and CD8+ lymphocyte subpopulations; STAT 1, 4 and 6 expression in splenocytes was assessed by Western blot, and serum cytokine levels were measured by ELISA. Adoptive transfer of NKT cells pulsed with HCC-derived antigens (group A) and NKT cells from immunized donors (group E) resulted in complete disappearance of tumors within 4 weeks and attenuated weight loss (6.5% and 7% in groups A and E, respectively). In contrast, mice in groups B, C, and D developed large, necrotic tumors and severe weight loss (21%, 17% and 23% weight loss in groups B, C, and D, respectively). NKT/CD4 and CD8/CD4 ratios were significantly increased in groups A and E (12.3 and 17.6 in groups A and D, respectively, compared to 6.4, 4.8 and 5.6 in groups B, C and D, respectively, for the NKT/CD4 ratio; 41 and 19.8 in groups A and E, respectively, compared to 6.5, 11.8 and 3.2 in groups B, C, and D, respectively, for the CD8/CD4 ratio). Expression of the transcription factor STAT4 was evident in group A, but not in groups B-D. Serum IFNγ, IL12 and IL4 levels were increased in groups A and E. Adoptive transfer of NKT lymphocytes exposed *ex vivo* by HCC-derived antigens loaded on dendritic cells and NKT cells from immunized donors led to suppression of HCC in mice. NKT-mediated anti-tumor activity was associated increased NKT and CD8+ T lymphocyte numbers, increased expression of STAT4, a marker for IL-12 activity and elevated serum levels of the proinflammatory cytokines IFNγ and IL12, and of IL4. *Ex-vivo* modulation of NKT lymphocytes holds promise as a novel mode of immune therapy for HCC.

Key words: NKT lymphocytes; hepatoma

Hepatocellular carcinoma (HCC) is a chemotherapy- and radiotherapy-resistant tumor, accounting for about 1% of all cancers in Europe and North America. Although palliative treatments (including surgical resection, chemoembolization, radioablation and intratumor alcohol injection) have prolonged survival, the overall prognosis remains poor.[1]

Enhancement of the anti-tumor immune response towards malignancies is a goal of many immune-therapeutic regimens. Several immune-modulatory techniques have been employed for treatment of HCC in experimental models. Notably, as hepatocellular carcinoma arising in the context of chronic hepatitis B virus infection often expresses hepatitis B surface antigen (HBsAg), some of these methods have exploited HBsAg as an immunotherapeutic target.[2,3] Adoptive transfer of bone marrow and splenocytes from donors immunized against HBsAg was shown to suppress HCC in athymic mice.[4] Oral immune regulation with HCC lysate and HBV envelope proteins, and adoptive transfer of dendritic cells pulsed *ex vivo* with HCC lysate and HBV envelope proteins, also suppressed HCC in the same model.[5,6] In all of these cases, suppression of HCC was accompanied by a shift towards a Th1 type immune response.

Natural killer T (NKT) lymphocytes are a subpopulation of regulatory lymphocytes that were shown to have a modulatory role in various immune-mediated disorders.[7,8] *Ex-vivo* immune programming of NKT lymphocytes was recently shown to be a feasible method for antigen-specific immune modulation in immune-mediated experimental colitis.[9] Recent studies suggest that NKT lymphocytes are important in anti-tumor immunity.[10] Although the role of these cells in the context of HCC has not been addressed directly, increased levels of NKT lymphocytes were observed in animals in which immune-modulatory interventions led to tumor suppression.[4–6]

The aim of our study was to assess the possibility of treating hepatocellular carcinoma by transplantation of *ex-vivo* "immune programmed" NKT cells. Adoptive transfer of NKT lymphocytes that were exposed *ex vivo* to tumor-associated antigens led to effective suppression of hepatocellular carcinoma in a murine model.

Methods

Mice

Female immunocompetent (heterozygous) and athymic Balb/c mice were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were kept in laminar flow hoods in sterilized cages and were given irradiated food and sterile acidified water. Animal experiments were carried out according to the guidelines of the Hebrew University-Hadassah Institutional Committee for Care and Use of Laboratory Animals and with the committee's approval.

Cell cultures

The HBsAg-expressing human hepatoma cell line, Hep-3B, was grown in culture as monolayers, in medium supplemented with nonessential amino acids and 10% heat inactivated fetal bovine serum.

NKT lymphocyte isolation

NKT cells were prepared from immunocompetent Balb/C mice. Splenocytes were isolated and prepared as previously described.[9] In brief, spleens were crushed through a stainless mesh (size 60,

Abbreviations: CTL, cytotoxic T lymphocyte; GVHD, graft *vs.* host disease; LAL, Liver-associated-lymphocytes; STAT, signal transducer and activator of transcription.

Grant sponsor: The Israel Science Foundation Grant; Grant sponsor: The Roaman-Epstein Liver Research Foundation.

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Received 12 July 2004; Accepted after revision 9 November 2004
DOI 10.1002/ijc.20889
Published online 1 February 2005 in Wiley InterScience (www.interscience.wiley.com).

TABLE I – EXPERIMENTAL GROUPS

Group	Transplanted cell	*Ex-vivo* exposure
A	NKT	HCC lysate
B	NKT	Hep3B cell line
C	NKT	BSA
D	No transplantation	None
E	NKT	HBV immunized donor

Sigma Chemical Co., St Louis, MO), and the resulting cell suspension washed twice in cold PBS. Cells were resuspended in PBS and placed through a nylon mesh presoaked in PBS; unbound cells were collected. For splenocyte isolation, 20 ml of histopaque 1077 (Sigma Diagnostics, St. Louis, MO) were slowly placed under the cells. After centrifugation and collection of cells at the interface, viability was found to be $> 95\%$ by trypan blue staining. Approximately 1×10^8 cells/mouse were recovered. To isolate NKT cells, Magnetic Cell Sorting (MACS) with anti-CD3 and anti-DX5 microbeads (Miltenyi Biotec, Germany) was performed. It should be noted that anti-DX5 isolates both natural killer T lymphocytes and natural killer cells. In a preliminary FACS analysis, we have determined that 2.8% of splenocytes are DX5+, 1.6% of splenocytes (57% of DX5+ cells) are natural killer cells and 1.2% of splenocytes (43% of DX5+ cells) are natural killer T cells that concomitantly express CD3; anti-CD11c beads were used for separation of dendritic cells and underwent *in vitro* maturation.[6]

Ex-vivo *pulsing of NKT cells*

To prepare HCC lysate, Hep3B cells were mechanically homogenized. After filtration through a 40 μm nylon cell strainer, intact cells were spun down and removed. Proteins were quantified using a protein assay kit (Biorad, Munich, Germany). Isolated NKT cells were pulsed *ex vivo* for 48 hr in the presence of dendritic cells (1×10^4 DC/1×10^6 NKT cells) by 3 μg/ml HCC lysate (group A), 2.5×10^3 Hep3B cells (group B) or 3 μg/ml BSA (group C). Supernatants were analyzed for IL12, IFNγ and IL4 levels.

HBV immunization of donor mice

Group E mice received 3 intramuscular injections of HBV vaccine (HbsAg+PeS1+PreS2) 4 weeks, 2 weeks and 1 week prior to initiation of our study.

Experimental groups: adoptive transfer of immune regulated lymphocytes

To determine the *in-vivo* effect of *ex-vivo* immune programming of NKT lymphocytes, 5 groups of recipient mice ($n = 10$ per group) were studied. Athymic recipient mice were conditioned with sublethal irradiation (600 cGy) and injected subcutaneously with 10^7 cells from the Hep3B human hepatoma cell line, as previously described.[3] Three days after injection of tumor cells, mice were transplanted with 1×10^7 splenocytes from naïve donors, followed by transplantation of 1×10^6 NKT lymphocytes per mouse 7 days later (Table I). Group A mice were transplanted with NKT cells that were pulsed *ex vivo* by HCC-lysate; group B mice were transplanted with NKT cells that were exposed *ex vivo* to Hep3B cells; group C mice were transplanted with NKT cells that were pulsed *ex vivo* by BSA; group D mice did not undergo transplantation of NKT lymphocytes and group E mice underwent transplantation of NKT cells from HBV-immunized donors.

Follow-up of tumor growth

Mice were followed for 8 weeks. Biweekly measurements of tumor volume and body weight were performed.

FACS analysis for determination of CD4+, CD8+ and NKT lymphocyte subpopulations

Immediately after lymphocyte isolation, triplicates of 2–5$\times$ 10^4 cells/500 μl PBS were placed into Falcon 2052 tubes, incubated with 4 ml of 1% BSA for 10 min and centrifuged at 1,400 rpm for 5 min. Cells were resuspended in 10 μl FCS; for analysis of the different subsets of T lymphocytes, anti-CD3 antibodies were combined with anti-DX5, anti-CD4 or anti CD8 antibodies (Pharmingen, San Diego, CA). Analytical cell sorting was performed on 1×10^4 cells from each group with a fluorescence-activated cell sorter (FACSTAR plus, Becton Dickinson, Oxnard, CA). Only live cells were counted, and background fluorescence from nonantibody-treated lymphocytes was subtracted. Gates were set on forward- and side-scatters to exclude dead cells and red blood cells. Data was analyzed with the Consort 30 2-color contour plot program (Becton Dickinson) or the CELLQuest 25 program.

Cytokine secretion

Supernatant and serum samples were collected. Levels of IFNγ, IL4 and IL12 were measured by "sandwich" ELISA using commercial kits (Genzyme Diagnostics, MA), according to the manufacturer's instructions.

STAT protein expression

Expression of the transcription factors STAT 1, 4 and 6 in splenocytes was determined by Western blot analysis of splenocytes harvested from mice in groups A–D. Splenocytes (10×10^6) were lysed in 100 μl of lysis solution (Sigma Chemical Co.). Proteins (100 μg/lane) were resolved by electrophoresis on SDS-polyacrylamide (7.5%) gels, and electroblotted to nitrocellulose membranes (Schleicher & Scuell, Germany). Probing with a polyclonal rabbit anti-mouse antibody for the different tested STAT proteins (anti-STAT 1, 4 and 6 antibodies, Santa Cruz Biotechnology, Santa Cruz, CA) was followed by addition of horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA). All experiments were repeated twice.

Statistical analysis

Statistical analysis was performed using the Student's *t* test.

Results

Effect of adoptive transfer of ex-vivo *immune programmed NKT lymphocytes on tumor growth*

Adoptive transfer of NKT lymphocytes that were pulsed *ex vivo* by HCC lysate or NKT cells from immunized donors led to significant suppression of hepatocellular carcinoma. There was no evidence of tumor in group A and group E mice (Fig. 1*a*); in contrast, group B and C mice, transplanted with NKT lymphocytes that were exposed *ex vivo* to Hep3B cells or BSA, respectively, and group D mice, that were not transplanted with NKT cells, developed large, necrotic tumors with multiple metastases (Fig. 1*b*).

Effect of adoptive transfer of ex-vivo immune programmed NKT lymphocytes on body weight: Weight loss was significantly attenuated in group A and group E mice, which underwent transplantation of HCC lysate-pulsed NKT lymphocytes and NKT cells from immunized donors, respectively, compared to the other study groups. Average body weights at baseline were 25.0 ± 2.0 g, 25.7 ± 1.3 g, 25.7 ± 1.3 g, 26.3 ± 0.9 g and 26.1 ± 1.1 in groups A, B, C, D and E, respectively. After 6 weeks, group A and group E mice lost 8% and 7% of body weight, respectively, while in groups B, C and D, an average weight loss of 21%, 16%, and 23% of body weight occurred, respectively (Fig. 2, $p < 0.05$).

Effect of adoptive transfer of ex-vivo *immune programmed NKT lymphocytes on CD8+ T cells, CD8+/CD4+ and NKT/CD4+ lymphocyte ratios*

Ex-vivo immune programming of NKT lymphocytes by HCC-lysate had a marked effect on lymphocyte subpopulations (Table II). In groups A and E, which underwent transplantation of HCC lysate-pulsed NKT lymphocytes and NKT cells from

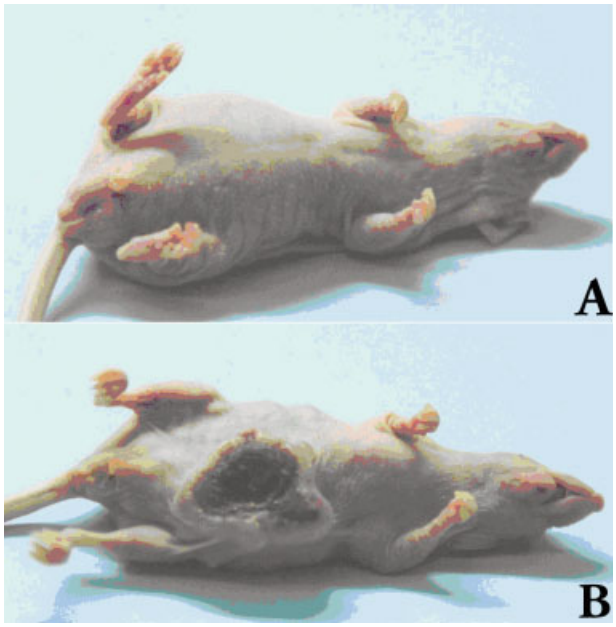

FIGURE 1 – Effect of adoptive transfer of *ex-vivo* immune programmed NKT lymphocytes on tumor growth: Representative animals from group A (*a*) and group B (*b*). Picture taken on day 54 following NKT lymphocyte transplantation.

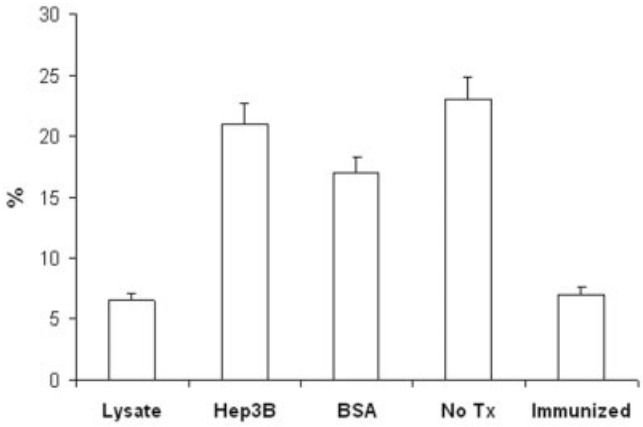

FIGURE 2 – Effect of adoptive transfer of *ex-vivo* immune programmed NKT lymphocytes on body weight: Percent of average weight loss between baseline and day 54 post transplantation ($n = 10$ per group).

immunized donors, respectively, the percentage of peripheral CD8+ T was significantly increased (53.4% and 82.8% in groups A and E, respectively, compared to 8.3%, 14.1% and 12.2% in groups B, C and D, respectively). The CD8+/CD4+ lymphocyte ratio, which was 41 and 19.8 in groups A and E, respectively, was significantly increased compared to values in groups B, C and D (6.5, 11.8 and 3.2, respectively, $p < 0.05$). Adoptive transfer of HCC lysate-pulsed NKT cells had a marked effect on the NKT lymphocyte ratio. The average NKT/CD4+ ratio was 12.3 and 17.6 in groups A and E, respectively, compared to 6.4, 4.8, and 5.6 in groups B, C and D, respectively ($p < 0.05$). Considering the relatively small number of transferred lymphocytes, it is unlikely that these differences represent a sole effect of transplanted cells.

Effect of adoptive transfer of *ex-vivo* immune programmed NKT lymphocytes on cytokine secretion: Adoptive transfer of HCC lysate-pulsed NKT cells led to significantly increased serum levels of the pro-inflammatory cytokines IFNγ and IL12. The serum IFNγ level was 97.3 pg/ml and 96.5 pg/ml in groups A and E, respectively, compared to 30.3, 55.1 and 67.8 pg/ml in groups B, C and D, respectively (Fig. 3*a*, $p < 0.05$ between groups A and D, and between B and D). The serum IL12 level was 842 pg/ml and 1,168 pg/ml in groups A and E, respectively, compared to 649, 737 and 722 pg/ml in groups B, C and D, respectively (Fig. 3*b*, $p < 0.05$ between groups A and D, and between B and D). Interestingly, adoptive transfer of HCC lysate-pulsed NKT cells also led to a significantly increased serum level of IL4; the serum IL4 level was 176 pg/ml and 202 pg/ml in groups A and E, respectively, compared to 15.1, 19.5 and 97.3 pg/ml in groups B, C and D, respectively (Fig. 3*c*, $p < 0.05$).

In vitro, supernatant IL12 was increased in group A (1,700 pg/ml) compared to group B and group C (800 pg/ml and 370 pg/ml, respectively) in the presence of dendritic cells but not in their absence; in the absence of dendritic cells, the serum IL12 level was low in all groups (Fig. 4). Supernatant IFNγ and IL4 levels were similar in groups A, B and C.

Effect of adoptive transfer of *ex-vivo* immune programmed NKT lymphocytes on expression of the transcription factors STAT 1, 4 and 6: STAT4 was expressed in group A but not in groups B–D (Fig. 5). STAT1 was expressed in groups A–C, but not in group D, that did not undergo NKT lymphocyte transplantation. STAT6 was expressed in all tested groups.

TABLE II – EFFECT OF ADOPTIVE TRANSFER OF *EX-VIVO* IMMUNE PROGRAMMED NKT LYMPHOCYTES ON CD8+ T CELLS, CD8+/CD4+ AND NKT/CD4+ LYMPHOCYTE RATIOS

Group	NKT (%)	CD4 (%)	CD8 (%)	CD8/CD4	NKT/CD4
A	16.0	1.3	53.4	41.0	12.3
B	8.1	1.3	8.3	6.5	6.4
C	5.8	1.2	14.1	11.8	4.8
D	20.8	3.7	12.2	3.2	5.6
E	73.5	4.2	82.3	19.8	17.6

Discussion

Transplantation of HCC lysate-pulsed NKT cells or NKT cells from HBV-immunized donors led to suppression of HCC and prevention of tumor metastasis in mice. Tumor suppression was associated with an increased percentage of peripheral CD8+ T lymphocytes, increased CD8+/CD4+ and NKT/CD4+ T lymphocyte ratios and increased serum levels of IFNγ, IL12, and IL4. Notably, similar patterns were observed in the 2 groups in which complete tumor regression occurred (groups A and E). In view of the diverse responses to transplantation of NKT cells that were exposed *ex vivo* to different pulsing regimens, the mere presence of additional NKT cells does not seem to be sufficient to suppress HCC. Thus, the environmental context in which NKT lymphocytes exert their effect appears to be significant.

NKT lymphocytes have a role in anti-tumor immunity.[10] Mouse and human NKT cells were shown exert cytotoxic activity towards several tumor cell lines.[11–13] A relative deficiency of NKT cells, defective NKT cell function or impaired presentation of tumor-associated antigens to NKT cells may jeopardize anti-tumor immunity and decreased numbers of intrahepatic NKT cells were found in tumor harboring mice.[14] NKT lymphocytes promote tumor rejection in experimental models of tumor immunotherapy by administration of IL12 or αGalCer.[15] Administration of αGalCer leads to rapid Th1 and Th2 cytokine production by NKT cells;[8] *in vivo*, αGalCer-induced IFNγ production by NKT cells, which may have a direct anti-tumor effect by itself, entails secondary natural killer cell activation, with further IFNγ production, increased cytotoxicity and proliferation and expression of

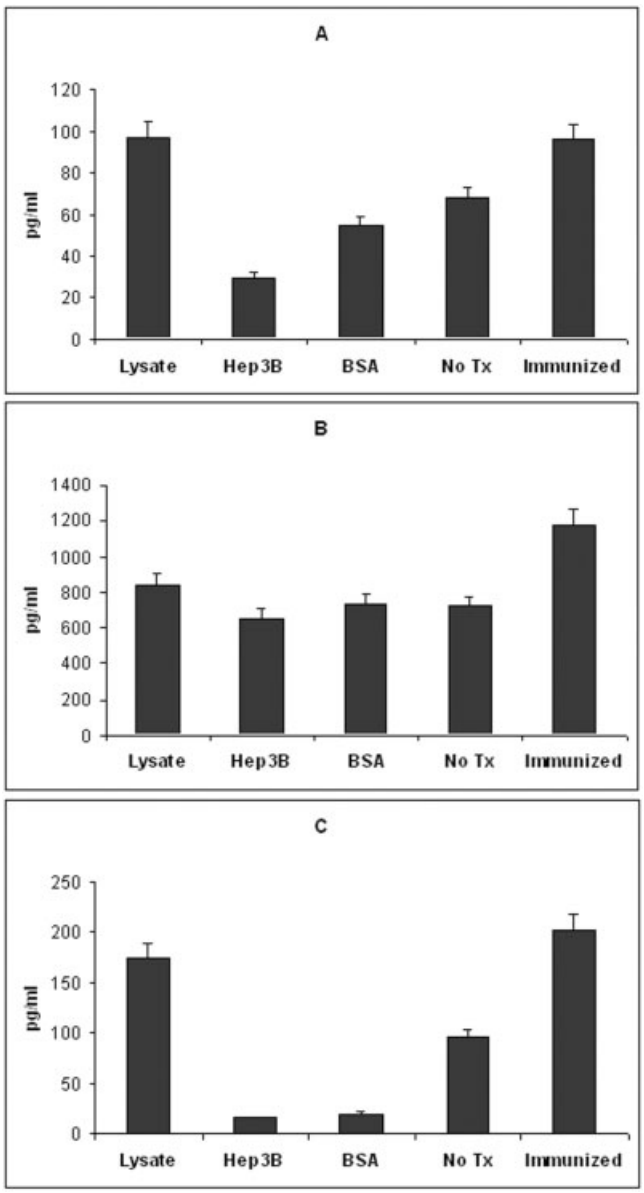

FIGURE 3 – Effect of adoptive transfer of *ex-vivo* immune programmed NKT lymphocytes on IFNγ (*a*), IL12 (*b*) and IL4 (*c*) cytokine serum levels. Serum samples were collected from mice in all groups on day 54 post transplantation and levels of IFNγ, IL4 and IL12 were measured by ELISA ($n = 10$ per group).

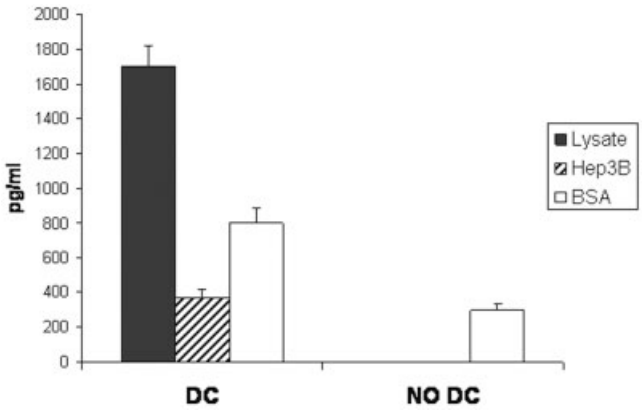

FIGURE 4 – Effect of adoptive transfer of ex-vivo immune programmed NKT lymphocytes on *in vitro* supernatant IL12 levels in the presence and absence of dendritic cells. To isolate NKT cells, Magnetic Cell Sorting (MACS) with anti-CD3 and anti-DX5 microbeads was performed. To prepare HCC lysate, Hep3B cells were mechanically homogenized. Isolated NKT cells were pulsed *ex vivo* for 48 hr in the presence of dendritic cells (1×10^4 DC/ $\times 10^6$ NKT cells) by 3 μg/ml HCC lysate (group A), 2.5×10^3 Hep3B cells (group B) or 3 μg/ml BSA (group C). Supernatants were analyzed for IL12 levels by ELISA. Results of triplicates are shown.

CD69.[16–] CD8+ T lymphocytes have also been implicated in the anti-tumor effect of αGalCer.[19] In humans, Vα24+ NKT cells, activated by α-GalCer, were shown to induce perforin-mediated killing of several tumor cell lines.[11] In contrast to these findings, it has been shown that in certain circumstances, NKT lymphocytes may suppress anti-tumor immunity. In 1 study, NKT lymphocytes and IL13, possibly produced by NKT cells and signaling through the IL4R-STAT6 pathway, were shown to downregulate tumor immunosurveillance.[20] In another study, NKT lymphocytes from UV-irradiated donor mice were found to suppress adaptive immune responses towards skin cancers *in vivo*.[21]

Thus, the role of NKT lymphocytes in immune-regulation is complex, and the shift mediated by these cells towards enhanced or suppressed immunity is often unpredictable; indeed, some have referred to these cells as a "double-edged sword" in the context of anti-tumor immunity.[22] The versatile behavior of NKT lymphocytes suggests that these cells are influenced by a variety of external stimuli, which may include different types of antigen presenting cells and costimulatory signals, soluble factors and effector cells within the microenvironment. Specific combinations of these elements (*i.e.*, activation of the NK1.1R or IL12R on NKT lymphocytes) may result in primarily Th1-type pro-inflammatory responses,[23,24] with secondary activation of NK cells and CTLs and enhanced anti-tumor responses. Alternatively, Th2-type anti-inflammatory responses, characterized by inhibition of CTLs and suppression of anti-tumor immunity, may occur.

In our study, a prominent anti-tumor effect in mice transplanted with NKT cells pulsed *ex vivo* by HCC lysate, or NKT cells from HBV-immunized donors (Groups A and E, respectively), was accompanied by increased peripheral CD8+ T lymphocyte numbers. While the number of CD4+ T lymphocytes did not differ significantly between the groups, the CD8/CD4 lymphocyte ratio in these mice was markedly elevated. Although the percentage of NKT lymphocytes was increased in groups A and E, in which tumor suppression occurred, in comparison to groups B and C, in which large tumors developed, the percentage of NKT lymphocytes was also relatively high (20.8%) in group D, which was not transplanted with NKT lymphocytes and in which no tumor suppression was observed. This finding suggests that the mere presence of higher numbers of NKT lymphocytes is not synonymous with an enhanced anti-tumor response. The relatively high number of NKT cells in group D also suggests that the percentage of NKT lymphocytes in the different study groups was unrelated to the administered inoculum of NKT cells.

NKT lymphocytes include subpopulations that are phenotypically and functionally diverse.[25] By itself, the percentage of peripheral NKT lymphocytes is insensitive to alterations in the relative proportion of different subpopulations of NKT cells, which may have contributed to the immune-modulatory effect observed in our study. While many NKT lymphocytes express CD4, others are double negative (CD4−CD8−) or CD8+. As the number of CD4+ T lymphocytes did not differ significantly between the study groups, the increased NKT/CD4+ T lymphocyte ratio in the groups in which tumor regression occurred may reflect an increase

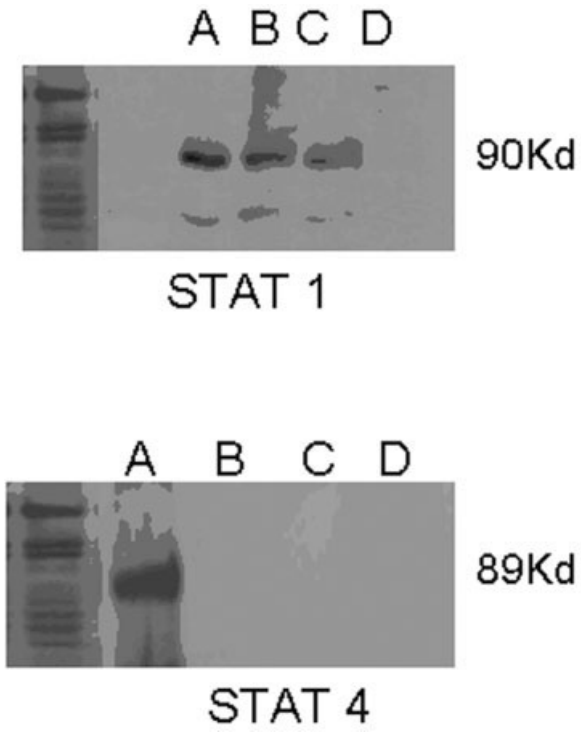

FIGURE 5 – Expression of transcription factors STAT 1 and 4 by Western blot analysis of splenocytes harvested from mice in groups A–D on day 54 following tranpslantation. Splenocytes (10×10^6) were lysed and proteins were resolved by electrophoresis on SDS-polyacrylamide gels. Probing with a polyclonal rabbit anti-mouse antibody for STAT 1 and 4 proteins was followed by addition of horseradish peroxidase-conjugated goat anti-rabbit IgG. Two repeated independent experiments were performed with identical results ($n = 10$ per group).

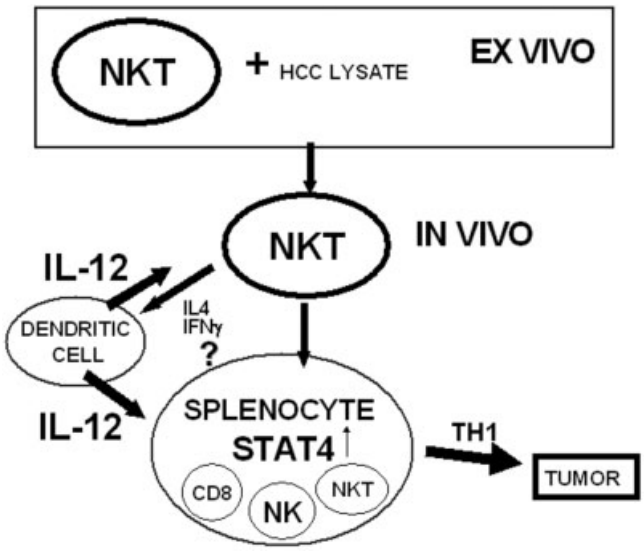

FIGURE 6 – Suggested mechanism for the effect of *ex-vivo* immune programmed NKT lymphocytes.

in the relative number of double negative and CD8+ NKT cells. Interestingly, expanded CD8+ NKT lymphocytes were recently shown to have a potent anti-leukemia effect.[26]

In addition to increased serum levels of the proinflammatory cytokines IFNγ and IL12, an increased level of serum IL4 was observed in groups A and E, in which tumor suppression occurred. As activated NKT lymphocytes secrete both IFNγ and IL4, the latter may have originated in activated NKT cells. Secretion of IL4 by NKT cells was shown to occur in a number of settings, in which NKT cells exert a proinflammatory effect. The anti-tumor effect of αGalCer was found to be associated with increased IL4 secretion.[27] Concanavalin A-induced hepatitis is a murine model of autoimmune hepatitis in which NKT lymphocytes are essential for the induction of liver damage; NKT cell-mediated liver injury does not occur in the absence of IL4.[28] It has been shown that elaboration of IL4 by NKT lymphocytes can lead to expansion and activation of type-1 dendritic cells (DC1)[29] and to secretion of bioactive IL12 by both murine and human dendritic cells,[30] contributing in effect to enhanced Th1 type immunity. IL4 may also enhance the anti-tumor activity of various effector cell subsets directly.

IL12, produced primarily by antigen presenting cells, is considered to have a key role in promotion of pro-inflammatory, Th1-type responses towards tumors and certain infectious agents (*i.e.*, Toxoplasma gondii, Mycobacterium tuberculosis).[31] Activation of the IL12 receptor leads to expression of the IL-12 specific transcription factor, STAT4. IL12 deficiency and reduced expression of STAT4 were linked to tumorigenesis in humans.[32] A prominent finding in our study was the correlation between tumor regression in mice transplanted with NKT cells pulsed *ex vivo* by HCC lysate (Group A) and expression of STAT4 in splenocytes. In contrast to serum cytokine levels, STAT expression by splenocytes reflects stimuli present in the immediate microenvironment of effector cells that are involved in anti-tumor immunity. Activated NKT lymphocytes secrete large amounts IFNγ and IL4, associated with expression of STAT1 and STAT6, respectively. In our study, no correlation was found between STAT1 or STAT6 expression and tumor suppression. This finding suggests that another, yet unidentified, factor, related to the transplantation of HCC lysate-pulsed NKT lymphocytes, led to increased IL12 secretion by antigen presenting cells with secondary activation of a Th1-type effector response and resultant tumor suppression (Fig. 6). *In vitro*, supernatant levels of IL12 were elevated in group A compared to groups B–D in the presence of dendritic cells but not in their absence. This finding may suggest that, in the presence of HCC lysate, NKT lymphocytes and dendritic cells are sufficient to induce the IL12-mediated anti-tumor response. The mechanism by which exposure to HCC lysate leads to enhanced IL12 production by dendritic cells remains unclear but seems to be related to the cross-talk between dendritic cells and NKT lymphocytes; apparently, presentation of a critical antigen by dendritic cells to NKT lymphocytes initiates a cytokine and costimulatory molecule-mediated dialogue that results in tumor suppression.

Malignancy-harboring hosts are known to have immune derangements that impair anti-tumor immunity, despite the presence of a large tumor antigen load.[33] Exposure to disease-associated antigens *ex vivo* is one method of bypassing this hurdle; in addition to overcoming the *in vivo* defect, this method enables to achieve optimal control over the pulsing conditions and to employ pulsing regimens that would raise safety and toxicity concerns in the *in vivo* setting. Interestingly, exposure of NKT lymphocytes to Hep3B cells (Group B) did not lead to tumor suppression. One possible explanation for the difference in the anti-tumor response towards whole or mechanically homogenized preparations of the same cells may be that a broader range of antigens (*i.e.*, intracellular antigens) was available in the HCC lysate pulsing medium. Alternatively, Hep3B cells may elaborate a yet-unidentified factor that suppresses anti-tumor immunity, stressing once again the advantage of *ex-vivo* pulsing with selected tumor antigens. As Hep3B cells express HBsAg, determination of the anti-tumor

effect of *ex-vivo* pulsing by HBsAg may help to differentiate between these possibilities.

Therapeutically, an important goal would be to develop a method of autologous transplantation of NKT lymphocytes, whereby these cells will be isolated, pulsed *ex vivo* by disease associated antigens and returned to a disease-harboring host. While transplantation of nonautologous splenocytes entails all drawbacks of allogeneic bone marrow transplantation, including GVHD, adoptive transfer of allogeneic NKT lymphocytes may have certain advantages in this setting. First, in our study, we have shown that transplantation of a relatively small number of NKT lymphocytes (1×10^6, an order of magnitude less than the number employed in BMT) was sufficient to mediate effective anti-tumor immunity. The requirement for small numbers of NKT lymphocytes will facilitate future clinical implementation of NKT cell mediated therapeutic regimens and can be expected to require relatively mild degrees of host conditioning. Another important advantage of transplantation of allogeneic NKT lymphocytes, rather than conventional BMT, relates to the fact that these cells do not cause GVHD.[34]

Although the Hep3B model employed in our study is a well-accepted experimental model for hepatoma, some of its characteristics do not enable to determine the feasibility of *ex-vivo* manipulation of autologous NKT lymphocytes. Hep3B is a human hepatoma cell line; to prevent rejection of tumors by a xeno-immune anti-human response, recipients have to be sublethally irradiated athymic animals. After inoculation with Hep3B cells, immune-competence is restored by transplantation of naïve splenocytes from syngeneic Balb/c donors, which are also the source for the NKT lymphocytes that undergo *ex-vivo* immune manipulation. Thus, isolation of immune cells from a tumor-bearing host cannot be achieved in this model. Although it may be suggested that enhancement of a xeno-immune response underlies tumor rejection in our study, as tumor-harboring mice were reconstituted with naïve splenocytes from immunocompetent donors, this possibility is unlikely in view of the lack of tumor inhibition in control groups that were treated by identical transplantation regimens.

In summary, in our study, adoptive transfer of small numbers of NKT cells pulsed *ex vivo* with HCC-derived antigens and NKT lymphocytes from HBV-immunized donors led to suppression of HCC in mice. This effect was associated with increased NKT and CD8 lymphocyte numbers, increased expression of STAT4, a marker for IL-12 activity and elevated serum IL12, IFNγ and IL4 levels. *Ex-vivo* manipulation of NKT lymphocytes enabled to achieve an anti-tumor immune response that did not develop spontaneously in tumor-harboring hosts. These findings suggest that transplantation of appropriately stimulated NKT lymphocytes may be a valuable addition to the developing arsenal of immunotherapeutic measures for the treatment of HCC and other malignancies.

Acknowledgements

Y.L. was supported by The Roaman-Epstein Liver Research Foundation.

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