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NKT Cells from Normal and Tumor-Bearing Human Livers Are Phenotypically and Functionally Distinct from Murine NKT Cells¹

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A major group of murine NK T (NKT) cells express an invariant V α 14J α 18 TCR α -chain specific for glycolipid Ags presented by CD1d. Murine V α 14J α 18⁺ account for 30–50% of hepatic T cells and have potent antitumor activities. We have enumerated and characterized their human counterparts, V α 24V β 11⁺ NKT cells, freshly isolated from histologically normal and tumor-bearing livers. In contrast to mice, human NKT cells are found in small numbers in healthy liver (0.5% of CD3⁺ cells) and blood (0.02%). In contrast to those in blood, most hepatic V α 24⁺ NKT cells express the V β 11 chain. They include CD4⁺, CD8⁺, and CD4⁻ CD8⁻ cells, and many express the NK cell markers CD56, CD161, and/or CD69. Importantly, human hepatic V α 24⁺ T cells are potent producers of IFN- γ and TNF- α , but not IL-2 or IL-4, when stimulated pharmacologically or with the NKT cell ligand, α -galactosylceramide. V α 24⁺V β 11⁺ cell numbers are reduced in tumor-bearing compared with healthy liver (0.1 vs 0.5%; $p < 0.04$). However, hepatic cells from cancer patients and healthy donors release similar amounts of IFN- γ in response to α -galactosylceramide. These data indicate that hepatic NKT cell repertoires are phenotypically and functionally distinct in humans and mice. Depletions of hepatic NKT cell subpopulations may underlie the susceptibility to metastatic liver disease. *The Journal of Immunology*, 2003, 171: 1775–1779.

Natural killer T (NKT) lymphocytes share receptor structures and functions of classical T cells and NK cells (1, 2). In mice the majority of NKT cells express a TCR consisting of an invariant α -chain, V α 14J α 18 (formally V α 14 α 281), and one of a limited number of β -chains that recognizes glycolipid Ags presented by the MHC class I-like protein CD1d (1–4). Humans appear to have a more heterogeneous repertoire of NKT cells that can express $\alpha\beta$ or $\gamma\delta$ TCR and NK cell markers (5). They include classical MHC-restricted T cells (5, 6) as well as CD1d-reactive NKT cells, also known as invariant NKT cells because of their invariant TCR α -chain rearrangement, V α 24J α 18 (formerly V α 24J α Q), and limited β -chain (V β 11) usage (7, 8). V α 24J α 18⁺ NKT cells display structural and functional homology to murine V α 14J α 18⁺ NKT cells (9). They can recognize the α -anomeric glycolipid α -galactosylceramide (α GalCer)⁴ and glycosylphosphatidylinositols in a CD1d-restricted manner (9–13). They can kill a variety of tumor cells (12, 14, 15) and upon

TCR stimulation can rapidly produce large amounts of IFN- γ and IL-4 (8, 16).

V α 14J α 18⁺ NKT cells appear to play a key role in antitumor defense in mice. Injection of mice with either IL-12 or α GalCer results in tumor rejection by a mechanism that is dependent upon IFN- γ production and/or antitumor cytotoxicity by NKT cells (17, 18). Furthermore, mice deficient in NKT cells fail to mediate IL-12-induced rejection of tumors (19). Because of the more heterogeneous nature of NKT cells in humans, few studies to date have addressed their role in antitumor immunity in this species. Invariant V α 14J α 18⁺ NKT cells constitute 30–50% of murine intrahepatic lymphocytes (1, 2, 20). In contrast, human liver contains only small numbers of V α 24⁺ NKT cells (21, 22), and the proportion of these cells that express the invariant V α 24J α 18 and V β 11 TCR chains is undetermined. However, T cells expressing the NK receptors CD56, CD161, CD94, and killer Ig-like receptors (KIR), although comprising small proportions of circulating lymphocytes, are substantially enriched in adult human liver (22, 23). In the present study we have enumerated and phenotypically and functionally characterized V α 24⁺V β 11⁺ NKT cells from freshly isolated liver specimens taken from healthy donors and from patients with hepatic malignancy. Our results indicate that human hepatic NKT cells are phenotypically and functionally more diverse than murine NKT cells, with only a minor proportion expressing invariant TCR chains. Hepatic V α 24V β 11⁺ T cells were found to produce Th1 (IFN- γ and TNF- α), but not Th2 (IL-4), cytokines. Their numbers are significantly lower in patients with hepatic malignancy, suggesting that these cells, like murine V α 14J α 18⁺ NKT cells, have antitumor roles in vivo.

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⁴ Abbreviations used in this paper: α GalCer, α -galactosylceramide; DN, double-negative; CD4⁻CD8⁻ phenotype; HMC, hepatic mononuclear cells; KIR, killer Ig-like receptors.

Materials and Methods

Tissue specimens

Wedge liver biopsies (50–100 mg) were obtained from seven healthy donor organs at the time of liver transplantation. Liver biochemistry and histology were normal in all cases. Liver tissue was obtained from 10

patients undergoing resection for hepatic metastases of colonic origin. Wedge biopsies from tumor-bearing tissue were taken ~10 cm from the tumor margin and appeared histologically normal. Hepatic mononuclear cell suspensions (HMC) were prepared as described previously (24). Matched PBMC were prepared from each donor and patient by Lymphoprep (Nycomed, Oslo, Norway) density gradient centrifugation. Ethical approval for this study was obtained from the ethics committee at St. Vincent's University Hospital (Dublin, Ireland).

Flow cytometry

The following mAbs were obtained from BD Biosciences (Oxford, U.K.): IgG1, anti-CD3, anti-CD4, anti-CD8, anti-CD56, anti-CD161, anti-CD25, anti-CD69, anti-CD45RA, and anti-HLA DR. Anti-V α 24 and anti-V β 11 were obtained from Immunotech (Marseille, France). Cells were stained using specific mAb according to standard procedures (22) and were analyzed by flow cytometry using the FACScan CellQuest software (BD Biosciences, San Jose, CA).

Analysis of cytokine production

Freshly isolated HMC were cultured for 6 h with or without 10 ng/ml PMA plus 1 μ g/ml ionomycin in 24-well plates at a concentration of 1×10^6 cells/ml. Brefeldin A (10 μ g/ml) was added for the last 4 h. Cells were stained as described previously using cell surface anti-V α 24 and anti-CD3, and intracytoplasmic anti-IFN- γ , anti-IL-2, anti-TNF- α , or anti-IL-4 (BD Biosciences) and were analyzed by flow cytometry (23).

In vitro response to α GalCer

HMC (10^6) were cultured in 24-well tissue culture plates in the presence of α GalCer (Kirin Pharmaceutical Research Laboratory, Gunma, Japan), or vehicle as a control, without the addition of further APC. After 48 h culture supernatants were collected from each well. IFN- γ production by the stimulated HMC was assayed by ELISA according to the manufacturer's protocols (Quantikine; R&D Systems, Oxon, U.K.).

Statistical analysis

The Mann-Whitney *U* test was used to compare distributions of cell populations in different groups, and $p < 0.05$ was taken as significant.

Results

V α 24V β 11⁺ NKT cells accumulate in human liver

Murine liver contains large numbers (30–50% of CD3⁺ cells) of V α 14V β 11⁺ NKT cells (1, 2, 20). We used flow cytometry to determine the frequencies of human T cells that express TCR V α 24 and V β 11 chains in seven histologically normal donor livers and matched blood samples (Fig. 1A). Fig. 1B shows that there is no enrichment of V α 24⁺ T cells in normal human liver relative to blood, since this TCR chain was found to be expressed by a median of 0.61% of peripheral blood CD3⁺ cells and 0.75% of hepatic CD3⁺ cells. However, V α 24⁺V β 11⁺ NKT cells were preferentially expanded in normal human liver, accounting for a median of 0.48% of hepatic CD3⁺ cells compared with 0.018% of peripheral blood T cells ($p = 0.02$; Fig. 1, A and C). Whereas a minority (2.9%) of V α 24⁺ T cells in blood expressed the V β 11 chain, up to 90% (median, 64.2%; $p = 0.04$) of the V α 24⁺ NKT cells in the liver were V β 11⁺.

Phenotypic characterization of hepatic V α 24⁺ NKT cells

Several studies have examined the phenotypes and functions of subpopulations of human NKT cells following stimulation in vitro with α GalCer (9, 10, 12, 13). Here we have examined the phenotypic and activation status of V α 24⁺ NKT cells ex vivo in the absence of pharmacological manipulation. The phenotypes of non-diseased hepatic V α 24⁺ NKT cells were compared with those of V α 24⁺ cells derived from blood from the same individuals (Fig. 2). CD4 and CD8 were expressed by significant numbers of peripheral and hepatic V α 24⁺ T cells, but while peripheral NKT cells displayed a predominance of CD4⁺ over CD8⁺ and double-negative (DN) CD4⁻CD8⁻ phenotypes, the majority of hepatic V α 24⁺ cells were CD8⁺ (28.3%) or DN (28.6%; Fig. 2B). A small

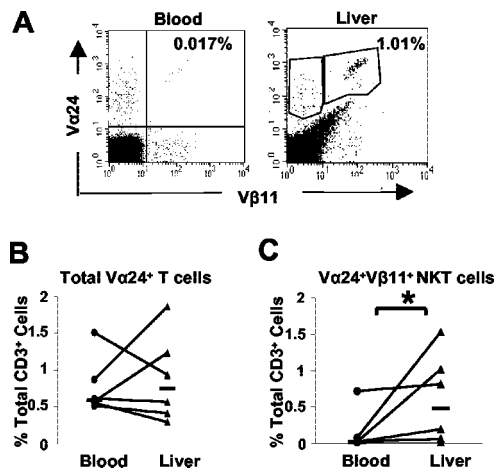


FIGURE 1. Small numbers of invariant V α 24V β 11⁺ NKT cells accumulate in human liver. *A*, Representative flow cytometry dot plot showing V α 24 and V β 11 TCR chain expression by gated CD3⁺ cells freshly isolated from blood and liver of a liver transplant donor. Numbers show percentages of CD3⁺ cells that express the V α 24V β 11 TCR. *B* and *C*, Percentages of CD3⁺ cells in blood and liver of six liver transplant donors expressing V α 24 (*B*) and V α 24V β 11 (*C*) TCR chains. Horizontal lines indicate medians. *, $p = 0.02$.

minority of circulating V α 24⁺ T cells expressed the NK markers CD56 (median, 3.3% of the total V α 24⁺ T cells) or CD161 (10.0%), while significantly higher proportions of hepatic V α 24⁺ T cells expressed CD56 (42.9%; $p = 0.03$) and CD161 (73.7%; $p = 0.02$). Analysis of activation status indicated that only a minority of hepatic V α 24⁺ NKT cells expressed CD25 (median, 5.7%), HLA-DR (15.5%), and CD45RA (7.3%), which are not significantly different from the frequencies of peripheral blood V α 24⁺ T cells expressing these markers. Significantly higher proportions of hepatic V α 24⁺ T cells expressed CD69 (median, 60.1%) compared with peripheral V α 24⁺ T cells (8.3%; $p = 0.03$; Fig. 2).

Decrease in invariant NKT cells in tumor-bearing liver

Decreased numbers of peripheral blood invariant NKT cells in patients with prostate cancer and melanoma have previously been reported (25, 26). We used flow cytometry to quantify V α 24⁺ and V α 24V β 11⁺ T cells among HMC taken from patients undergoing resection for hepatic malignancy. No significant decrease was seen between normal and tumor-bearing liver in terms of total V α 24⁺ T cells (0.75 vs 0.41% of CD3⁺ T cells; $p = 0.23$; Fig. 3A). However, the proportion of CD3⁺ cells expressing V α 24V β 11 TCR was significantly reduced in tumor-bearing livers compared with healthy livers (median, 0.098 vs 0.48%; $p = 0.04$; Fig. 3A).

The proportions of hepatic V α 24⁺ T cells that expressed CD4⁺, CD8⁺, or DN phenotypes were not significantly different between normal and tumor-bearing livers. CD56⁺ and CD161⁺ V α 24⁺ T cells were significantly decreased in tumor-bearing tissue ($p = 0.05$ and 0.03 , respectively), while the proportions of HLA-DR⁺ V α 24⁺ T cells were increased ($p = 0.03$; Fig. 3B). The majority of V α 24⁺ T cells in normal and tumor-bearing tissues were CD45RA⁻ and CD69⁺ (Fig. 3B).

Hepatic NKT cells predominantly produce Th1 cytokines

NKT cell lines and clones are known to rapidly produce large amounts of both Th1 and Th2 cytokines (8, 16). We used flow cytometry to examine the cytokine secretion profiles of human hepatic V α 24⁺ NKT cells in response to in vitro stimulation with

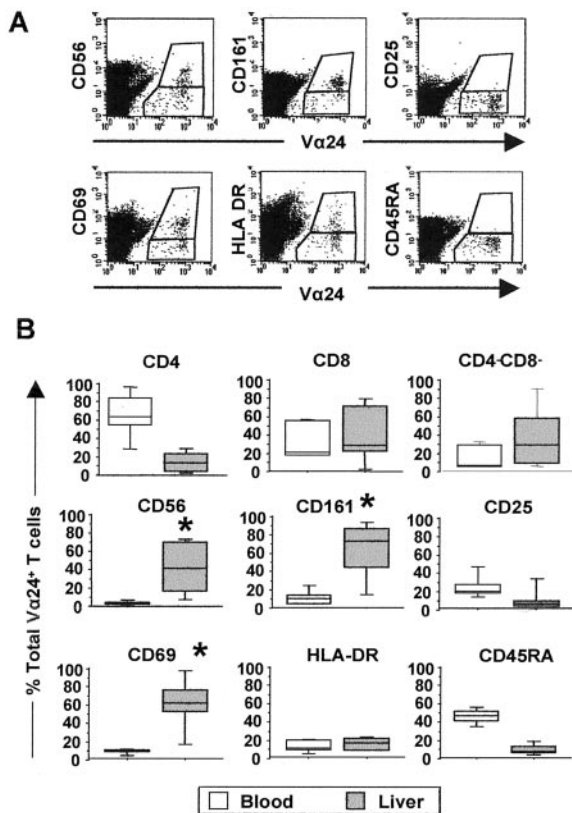


FIGURE 2. Phenotypic characterization of human hepatic and peripheral blood $V\alpha 24^+$ T cells. *A*, Representative flow cytometry dot plot showing CD56, CD161, CD25, CD69, HLA-DR, and CD45RA expression by hepatic $V\alpha 24^+$ T cells from a liver transplant donor. *B*, Box plots showing median (horizontal lines), interquartile ranges (shaded areas), and ranges (error bars) of percentages of $V\alpha 24^+$ T cells in blood and liver of six liver transplant donors expressing CD4⁺, CD8⁺, and CD4⁻CD8⁻ phenotypes, CD56, CD161, CD25, CD69, HLA-DR, and CD45RA. *, $p = 0.03$ for CD56, $p = 0.02$ for CD161, and $p = 0.03$ for CD69.

PMA and ionomycin (Fig. 4A). The majority of $V\alpha 24^+$ T cells from normal livers produced the inflammatory cytokines, IFN- γ (median, 83.2%) and TNF- α (54.7%) after stimulation (Fig. 4). A minority (4.26%) produced IL-2. While up to 4% of fresh PBMC expressed IL-4 upon stimulation with PMA and ionomycin (data not shown), IL-4 expression by hepatic $V\alpha 24^+$ T cells was not detectable (Fig. 4A). No differences were observed in the cytokine secretion profiles of hepatic $V\alpha 24^+$ T cells from healthy donors and patients with malignancy (Fig. 4B).

Hepatic response to α GalCer stimulation

Since invariant NKT cells were found to be present in such low numbers in both normal and tumor-bearing liver, we aimed to determine whether there was a measurable response to α GalCer stimulation in vitro. Freshly isolated HMC and matched PBMC from healthy donors and patients with hepatic malignancy were incubated for 48 h with α GalCer, and supernatants were assayed for IFN- γ and IL-4 production by ELISA. Culture in the presence of α GalCer caused a significant increase in the production of IFN- γ by HMC (389 pg/ml; Fig. 5) without the need for additional APC such as monocyte-derived dendritic cells (12, 13). This increase was not seen when PBMC were treated similarly (27 pg/ml). No IL-4 was detectable in the supernatants of α GalCer-stimulated HMC, even though IL-4 was readily detected in PHA-stimulated HMC or PBMC (Fig. 5). HMC from patients with hepatic malig-

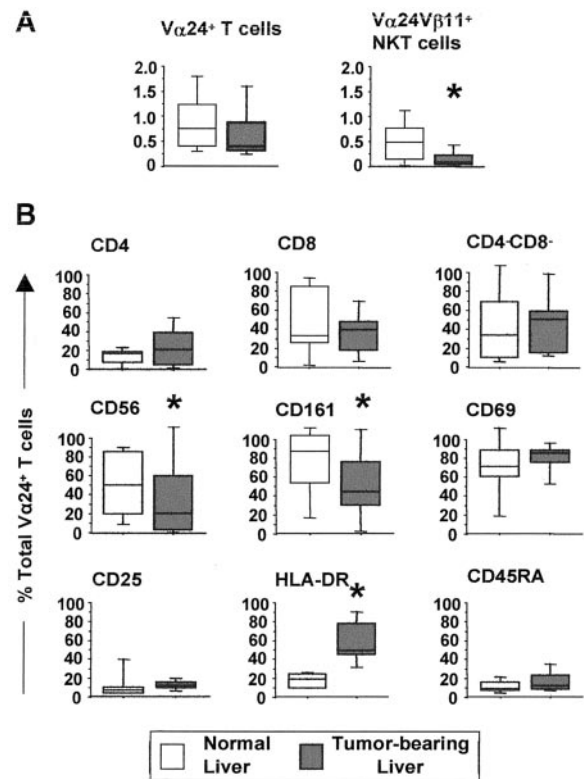


FIGURE 3. Invariant NKT cells are selectively depleted in tumor-bearing liver. *A*, Box plots showing median (horizontal lines), interquartile ranges (shaded areas), and ranges (error bars) of percentages of CD3⁺ cells in normal donor and tumor-bearing liver expressing $V\alpha 24$ TCR chain and $V\alpha 24V\beta 11$ TCR. *, $p = 0.04$. *B*, Box plots showing median, interquartile ranges, and ranges of percentages of hepatic $V\alpha 24^+$ T cells in six normal donor livers and nine tumor-bearing livers expressing CD4⁺, CD8⁺, and CD4⁻CD8⁻ phenotypes, CD56, CD161, CD25, CD69, HLA-DR, and CD45RA. *, $p = 0.05$ for CD56, $p = 0.03$ for CD161, and $p = 0.03$ for HLA-DR.

nancy secreted levels of IFN- γ similar to those of normal HMC after stimulation with α GalCer (407 pg/ml; Fig. 5).

Discussion

There are large numbers of T cells in healthy liver, and over one-third of these express NK receptors (22, 23). In mice, the majority of NK receptor-positive hepatic T cells express invariant $V\alpha 14^+J\alpha 18$ TCRs, which recognize glycolipid Ags presented by CD1d and appear to have important antimetastatic roles (1–4, 17–19). Human NKT cells are phenotypically and functionally more diverse than their murine counterparts, and in the absence of definable markers for human NKT cells, little is known about their potential antimetastatic functions. About one-third of human hepatic T cells express NK markers and exhibit antitumor cytotoxicity and rapid cytokine secretion, similar to murine NKT cells (22, 23). Studies of human $V\alpha 24J\alpha 18^+$ NKT cells have relied on the use of in vitro-generated lines and clones, and it is now well established that α GalCer-stimulated $V\alpha 24J\alpha 18^+$ NKT cells are structurally and functionally homologous to murine $V\alpha 14J\alpha 18^+$ NKT cells (1, 2, 8, 9, 12).

In the present study we enumerate $V\alpha 24V\beta 11^+$ NKT cells in unmanipulated, freshly isolated normal liver and matched blood specimens and show that they account for very low proportions (~0.02% in blood and 0.5% in liver) of the total T cell compartment. This compares with frequencies of ~4% of peripheral and 30–50% of hepatic T cells that express the homologous $V\alpha 14J\alpha 18$

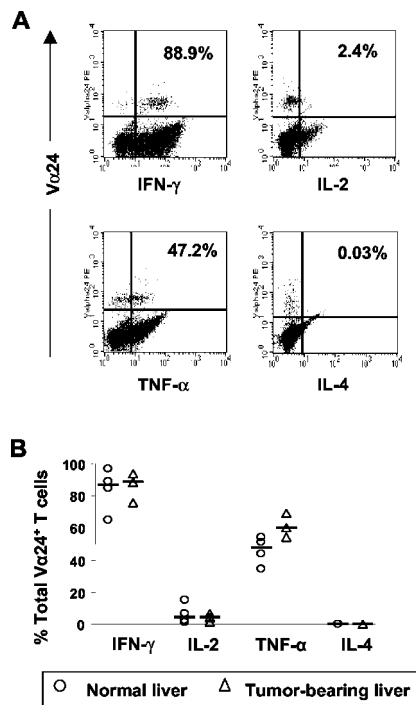


FIGURE 4. Hepatic NKT cells predominantly produce Th1 cytokines upon stimulation *ex vivo*. **A**, Representative flow cytometry dot plot showing IFN- γ , IL-2, TNF- α , and IL-4 expression by hepatic V α 24⁺ T cells stimulated for 4 h with PMA and ionomycin. The percentages of V α 24⁺ cells that express the cytokines are indicated in the *upper right quadrants*. **B**, Median percentages of V α 24⁺ cells in four normal donor livers and four tumor-bearing livers that express IFN- γ , IL-2, TNF- α , and IL-4 upon stimulation.

TCR in mice (1, 2, 20). In contrast to those in blood, the majority of human hepatic V α 24⁺ cells express the V β 11 chain associated with the invariant V α 24J α 18 TCR of NKT cells. Significant proportions of unstimulated hepatic V α 24⁺ cells express CD4⁺, CD8⁺, DN, CD56⁺, CD161⁺, CD69⁺, and CD45RA⁻ phenotypes, similar to those of α GalCer-stimulated human NKT cells (10, 12, 13, 15, 18), suggesting that these cells may have previously encountered a natural ligand within the liver. However, in contrast to NKT cells generated by culture *in vitro*, which are potent producers of IL-4, the majority of freshly isolated V α 24⁺ T cells exhibited a striking bias toward Th1 cytokine production, producing IFN- γ and TNF- α , but not IL-4, in response to stimulation with phorbol ester or α GalCer. Recent studies of α GalCer-stimulated human NKT cells (12, 13) and PBMC that stain positively for CD1d- α GalCer tetramers (27, 28) have provided evidence that only the CD4⁺ subset of NKT cells can produce both IFN- γ and IL-4, while DN and CD8⁺ NKT cells produce Th1 cytokines only. We show here that human hepatic NKT cells only exhibit Th1 cytokine phenotypes even though they include significant numbers of CD8⁺, CD4⁺, and DN cells. Human hepatic V α 24⁺ NKT cells with a predominant Th1 cytokine bias have also been described in patients with chronic hepatitis C infection (29).

Although invariant V α 24V β 11⁺ NKT cells, known to recognize glycolipids such as α GalCer in the context of CD1d (8–10), constitute a small proportion of human hepatic T cells, we have shown that significant levels of IFN- γ are produced by HMC after stimulation with α GalCer. While it is likely that some of this IFN- γ is produced by cells downstream from NKT cells, such as NK cells and CTLs (14, 30), this finding suggests that other non-invariant CD1d-reactive NKT cells may be present in the liver.

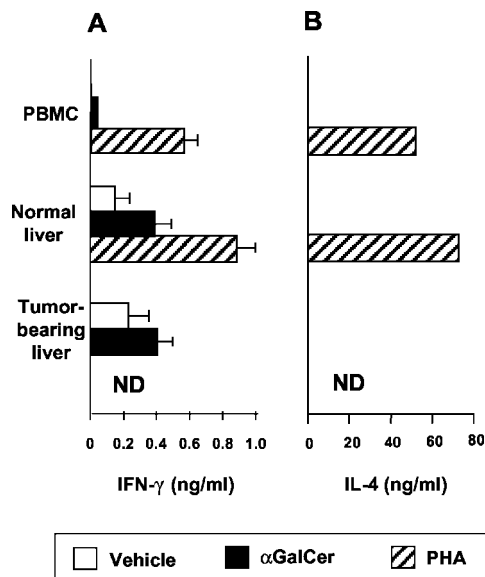


FIGURE 5. HMC response to α GalCer stimulation. Mean IFN- γ (**A**) and IL-4 (**B**) levels released by vehicle-, α GalCer-, and PHA-stimulated MNC isolated from blood and livers of five liver transplant donors and from three livers of patients with hepatic malignancy. IL-4 production by α GalCer-stimulated HMC was not detected using ELISA sensitive to 1 pg/ml. ND, PHA stimulation of tumor-bearing liver was not performed.

Noninvariant CD1d-restricted NKT cells that express CD56 and/or CD161 have been detected in human bone marrow (31) and in hepatitis C virus-infected liver (29). However, recent studies (32, 33) have provided evidence that CD1d- α GalCer tetramers only stain V α 24V β 11⁺ NKT cells, which would argue against the idea of noninvariant hepatic NKT cells recognizing α GalCer. NKT cells reactive with CD1 isotypes that are not found in mice, namely CD1a, CD1b, and CD1c, may also reside within the CD56⁺/CD161⁺ T cell compartment of the liver (3, 4).

Our results further indicate that V α 24V β 11⁺ NKT cells, but not other V α 24⁺ cells, are found in significantly lower numbers in histologically normal portions of livers from patients with metastatic liver disease. This reduction in NKT cell numbers could predispose individuals to the development of malignancy or, alternatively, may be the result of activation-induced cell death of these putative antitumor effectors. A decrease in the numbers of NKT cells in peripheral blood has been reported in patients with advanced prostate cancer (25). Compared with controls, PBMC isolated from these patients exhibited diminished expansion of V α 24V β 11⁺ NKT cells in response to α GalCer and diminished IFN- γ production by the expanded NKT cells. In contrast, Kawano et al. (26) reported a decrease in V α 24⁺ NKT cell numbers in the blood of patients with melanoma, but these cells exhibited normal responses to α GalCer and normal levels of cytotoxicity. We found that V α 24V β 11⁺ NKT cells are reduced in the livers of patients with hepatic malignancy, but that freshly isolated hepatic V α 24⁺ T cells from healthy donors and patients with hepatic malignancy had similar frequencies of IFN- γ -producing cells. It should be noted that these V α 24⁺ T cells in the cancer patients include both V β 11⁺ NKT cells and V β 11⁻ non-NKT cells. However, fresh HMC from cancer patients and controls released similar levels of IFN- γ after stimulation with α GalCer *in vitro*. This suggests that invariant hepatic V α 24V β 11⁺ NKT cells from patients with hepatic malignancy release IFN- γ in response to α GalCer, but that downstream events may compensate for the reduction in NKT cell numbers in these patients.

In conclusion, we have demonstrated that the human liver contains small numbers of invariant $V\alpha 24V\beta 11^+$ NKT cells, but significant numbers of α GalCer-reactive NKT cells, suggesting that the repertoires of hepatic NKT cells are more diverse in humans than in mice. In contrast to in vitro-stimulated murine and human peripheral invariant NKT cells (1, 2, 8, 16), freshly isolated human hepatic $V\alpha 24V\beta 11^+$ NKT cells do not produce IL-2 and IL-4 upon stimulation. Depletions of hepatic $V\alpha 24V\beta 11^+$ cells, but not other $V\alpha 24^+$ or α GalCer-reactive cells, are likely to predispose individuals to metastatic liver disease. However, the differences in numbers, TCR specificities, and functions of hepatic NKT cells in mice and humans need to be taken into account when interpreting the roles of these cells in experimental models of malignancy.

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