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/content/171/10/5631.1.full.pdf
NKT Cells from Normal and Tumor-Bearing Human Livers Are Phenotypically and Functionally Distinct from Murine NKT Cells

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A major group of murine NK T (NKT) cells express an invariant Vα14Jα18 TCR α-chain specific for glycolipid Ags presented by CD1d. Murine Vα14Jα18+ account for 30–50% of hepatic T cells and have potent antitumor activities. We have enumerated and characterized their human counterparts, Vα24Vβ11+ 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 Vα24+ NKT cells express the Vβ11 chain. They include CD4+, CD8+, and CD4−CD8− cells and many express the NK cell markers CD56, CD161, and/or CD69. Importantly, human hepatic Vα24+ 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. Vα24+ Vβ11+ 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, Vα14Jα18 (formally Vα14Jα281), 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 αβ or γδ 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, Vα24Jα18 (formerly Vα24JαQ), and limited β-chain (Vβ11) usage (7, 8). Vα24Jα18+ NKT cells display structural and functional homology to murine Vα14Jα18+ NKT cells (9). They can recognize the α-anomeric glycolipid α-galactosylceramide (αGalCer) and glycosylphosphatidylinositol 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).

Vα14Jα18+ NKT cells appear to play a key role in antitumor defense in mice. Injection of mice with either IL-12 or αGalCer 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 Vα14Jα18+ NKT cell constitute 30–50% of murine intrahepatic lymphocytes (1, 2, 20). In contrast, human liver contains only small numbers of Vα24− NKT cells (21, 22), and the proportion of these cells that express the invariant Vα24Jα18 and Vβ11 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 Vα24+Vβ11+ 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 Vα24Vβ11+ 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 Vα14Jα18+ NKT cells, have antitumor roles in vivo.

Materials and Methods

Tissue specimens

Wedge liver biopsies (50–100 mg) were obtained from seven healthy donors 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-Vα24 and anti-Vβ11 were obtained from Immunotech (Marseilles, France). Cells were stained using specific mAb according to standard procedures (22) and were analyzed by flow cytometry using the FACSScan 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 μg/ml ionomycin in 24-well plates at a concentration of 1 × 10^6 cells/ml. Brefeldin A (10 μg/ml) was added for the last 4 h. Cells were stained as described previously using cell surface anti-Vα24 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 αGalCer**

HMC (10^6) were cultured in 24-well tissue culture plates in the presence of αGalCer (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**

**Vα24Vβ11+ NKT cells accumulate in human liver**

Murine liver contains large numbers (30–50% of CD3+ cells) of Vα14Vβ11+ NKT cells (1, 2, 20). We used flow cytometry to determine the frequencies of human T cells that express TCR Vα24 and Vβ11 chains in seven histologically normal donor livers and matched blood samples (Fig. 1A). Fig. 1B shows that there is no enrichment of Vα24+ 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, Vα24+Vβ11+ 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 Vα24+ T cells in blood expressed the Vβ11 chain, up to 90% (median, 64.2%; p = 0.04) of the Vα24+ NKT cells in the liver were Vβ11+.

**Phenotypic characterization of hepatic Vα24+ NKT cells**

Several studies have examined the phenotypes and functions of subpopulations of human NKT cells following stimulation in vitro with αGalCer (9, 10, 12, 13). Here we have examined the phenotypic and activation status of Vα24+ NKT cells ex vivo in the absence of pharmacological manipulation. The phenotypes of non-diseased hepatic Vα24+ NKT cells were compared with those of Vα24+ cells derived from blood from the same individuals (Fig. 2). CD4 and CD8 were expressed by significant numbers of peripheral and hepatic Vα24+ 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 Vα24+ cells were CD8+ (28.3%) or DN (28.6%; Fig. 2B). A small minority of circulating Vα24+ T cells expressed the NK markers CD56 (median, 3.3% of the total Vα24+ T cells) or CD161 (10.0%), while significantly higher proportions of hepatic Vα24+ 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 Vα24+ 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 Vα24+ T cells expressing these markers. Significantly higher proportions of hepatic Vα24+ T cells expressed CD69 (median, 60.1%) compared with peripheral Vα24+ 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 Vα24+ and Vα24Vβ11+ 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 Vα24+ T cells (0.75 vs 0.41% of CD3+ T cells; p = 0.23; Fig. 3A). However, the proportion of CD3+ cells expressing Vα24Vβ11 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 Vα24+ T cells that expressed CD4+, CD8+, or DN phenotypes were not significantly different between normal and tumor-bearing livers. CD56+ and CD161+ Vα24+ T cells were significantly decreased in tumor-bearing tissue (p = 0.05 and 0.03, respectively), while the proportions of HLA-DR+ Vα24+ T cells were increased (p = 0.03; Fig. 3B). The majority of Vα24+ 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 Vα24+ NKT cells in response to in vitro stimulation with

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**FIGURE 1.** Small numbers of invariant Vα24Vβ11+ NKT cells accumulate in human liver. A, Representative flow cytometry dot plot showing Vα24 and Vβ11 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 Vα24Vβ11 TCR. B and C, Percentages of CD3+ cells in blood and liver of six liver transplant donors expressing Vα24 (B) and Vα24Vβ11 (C) TCR chains. Horizontal lines indicate medians. *p = 0.02.
PMA and ionomycin (Fig. 4A). The majority of Vo24+ 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 Vo24+ T cells was not detectable (Fig. 4A). No differences were observed in the cytokine secretion profiles of hepatic Vo24+ T cells from healthy donors and patients with malignancy (Fig. 4B).

**Hepatic response to αGalCer 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 αGalCer stimulation in vitro. Freshly isolated HMC and matched PBMC from healthy donors and patients with hepatic malignancy were incubated for 48 h with αGalCer, and supernatants were assayed for IFN-γ and IL-4 production by ELISA. Culture in the presence of αGalCer 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 αGalCer-stimulated HMC, even though IL-4 was readily detected in PHA-stimulated HMC or PBMC (Fig. 5). HMC from patients with hepatic malignancy secreted levels of IFN-γ similar to those of normal HMC after stimulation with αGalCer (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 Vα14+α18 TCRs, which recognize glycolipid Ags presented by CD1d and appear to have important antitumor cytotoxic and rapid cytokine secretion, similar to murine NKT cells (22, 23). Studies of human Vo24Jα18+ NKT cells have relied on the use of in vitro-generated lines and clones, and it is now well established that αGalCer-stimulated Vo24Jα18+ NKT cells are structurally and functionally homologous to murine Vα14Jα18+ NKT cells (1, 2, 8, 9, 12).

In the present study we enumerate Vo24Vβ11+ 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 Vα14Jα18
TCR in mice (1, 2, 20). In contrast to those in blood, the majority of human hepatic Vα24+ cells express the Vβ11 chain associated with the invariant Vα24β11 TCR of NKT cells. Significant proportions of unstimulated hepatic Vα24+ cells express CD4+, CD8+, DN, CD56+, CD161+, CD69+, and CD45RA− phenotypes, similar to those of αGalCer-stimulated human NKT cells (10, 12, 13), 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 Vα24+ 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 αGalCer. Recent studies of αGalCer-stimulated human NKT cells (12, 13) and PBMC that stain positively for CD1d-αGalCer 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 Vα24+ NKT cells with a predominant Th1 cytokine bias have also been described in patients with chronic hepatitis C infection (29).

Although invariant Vα24β11+ NKT cells, known to recognize glycolipids such as αGalCer 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 αGalCer. 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 noninvariant CD1d-reactive NKT cells may be present in the liver.

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-αGalCer tetramers only stain Vα24β11+ NKT cells, which would argue against the idea of noninvariant hepatic NKT cells recognizing αGalCer. 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 Vα24β11+ NKT cells, but not other Vα24+ 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 Vα24β11+ NKT cells in response to αGalCer and diminished IFN-γ production by the expanded NKT cells. In contrast, Kawano et al. (26) reported a decrease in Vα24+ NKT cell numbers in the blood of patients with melanoma, but these cells exhibited normal responses to αGalCer and normal levels of cytotoxicity. We found that Vα24β11+ NKT cells are reduced in the livers of patients with hepatic malignancy, but that freshly isolated hepatic Vα24+ T cells from healthy donors and patients with hepatic malignancy had similar frequencies of IFN-γ-producing cells. It should be noted that these Vα24+ T cells in the cancer patients include both Vβ11+ NKT cells and Vβ11− non-NKT cells. However, fresh HMC from cancer patients and controls released similar levels of IFN-γ after stimulation with αGalCer in vitro. This suggests that invariant hepatic Vα24β11+ NKT cells from patients with hepatic malignancy release IFN-γ in response to αGalCer, 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 Vα24Vß11+ NKT cells, but significant numbers of αGalCer-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 Vα24Vß11+ NKT cells do not produce IL-2 and IL-4 upon stimulation. Depletions of hepatic Vα24Vß11+ cells, but not other Vα24+ or αGalCer-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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References

CORRECTIONS


The second author’s name is listed incorrectly. The correct name is Lucy Golden-Mason.


In *Materials and Methods*, in the first sentence under the heading *Ag processing and presentation assays*, the sequence of the control CpG oligodeoxynucleotide 1982 is incorrect. The correct sentence is below.

Nonmethylated, phosphorothioate-modified CpG ODN 1826 (TCCATGACGTTCTGACGTT) and non-CpG ODN 1982 (TCCAGGACTTCTCTCAGGTT) were generously provided by Coley Pharmaceutical Group (Ottawa, Ontario, Canada).


The last author’s name is reversed. The correct name is Nitza Lahat.