Enrichment of Human CD4$^+$ Vα24/Vβ11 Invariant NKT Cells in Intrahepatic Malignant Tumors[1]

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Invariant NKT cells (iNKT cells) recognize glycolipid Ags via an invariant TCR α-chain and play a central role in various immune responses. Although human CD4$^+$ and CD4$^-$ iNKT cell subsets both produce Th1 cytokines, the CD4$^+$ subset displays an enhanced ability to secrete Th2 cytokines and shows regulatory activity. We performed an ex vivo analysis of blood, liver, and tumor iNKT cells from patients with hepatocellular carcinoma and metastases from uveal melanoma or colon carcinoma. Frequencies of Vα24/Vβ11 iNKT cells were increased in tumors, especially in patients with hepatocellular carcinoma. The proportions of CD4$^+$, double negative, and CD8α^+ iNKT cell subsets in the blood of patients were similar to those of healthy donors. However, we consistently found that the proportion of CD4$^+$ iNKT cells increased gradually from blood to liver to tumor. Furthermore, CD4$^+$ iNKT cell clones generated from healthy donors were functionally distinct from their CD4$^-$ counterparts, exhibiting higher Th2 cytokine production and lower cytolytic activity. Thus, in the tumor microenvironment the iNKT cell repertoire is modified by the enrichment of CD4$^+$ iNKT cells, a subset able to generate Th2 cytokines that can inhibit the expansion of tumor Ag-specific CD8$^+$ T cells. Because CD4$^+$ iNKT cells appear inefficient in tumor defense and may even favor tumor growth and recurrence, novel iNKT-targeted therapies should restore CD4$^-$ iNKT cells at the tumor site and specifically induce Th1 cytokine production from all iNKT cell subsets. *The Journal of Immunology,* **2009, 182: 5140–5151.**

Invariant NKT (iNKT)[3] cells or type I NKT cells (1, 2) bear a semi-invariant $\alpha\beta$ TCR that is restricted by the nonpolymorphic MHC class I-like molecule CD1d. They are important in the regulation of various immune responses against infections, tumors, autoimmune diseases, and tolerance induction (3). These cells display reactivity to self-derived glycolipids presented by CD1d and strong reactivity to synthetic α-galactosylceramide (α-GalCer) (4). Murine iNKT cells have an invariant Vα14Jα18 chain that is paired with a limited number of β-chains (Vβ8, Vβ7, Vβ2) (1, 2). Human iNKT cells (type I NKT cells) bear a TCR with an invariant Vα24Jα18 chain paired with diverse Vβ11 chains (1, 2, 4, 5). There is a strong correlation between the usage of Vα24/Vβ11 TCR segments, expression of the invariant Vα24Jα18 chain (6, 7), and binding to α-GalCer-loaded CD1d tetramers (8–11). However, few Vβ11$^-$ human NKT cells expressing the invariant Vα24Jα18 chain have been reported (5), and some Vα24$^-$ T cells reactive to α-GalCer/CD1d have been described in PBMC after α-GalCer-mediated expansion in vitro (12). These Vα24$^-$ cells were rarely detectable ex vivo (12), and some of them were found to have a TCR rearranged with the Jα18 segment (13). Very recently, the new clonotypic mAb 6B11, specific for the Vα24Jα18 CDR3 loop, has been shown to selectively stain human iNKT cells (14, 15). Thus, Vα24Vβ11 staining is a good but surrogate marker for iNKT cells. The term iNKT cells used in this study refers exclusively to human semi-invariant NKT cells, defined as Vα24/Vβ11-expressing T cells (1, 2).

In both mouse and human, another class of CD1d-restricted $\alpha\beta$ T cells (type II NKT cells) use variable TCR segments and are not reactive to α-GalCer (1, 2). Although a majority of NK1.1$^+$ T cells in naive mice correspond to α-GalCer-reactive iNKT cells (1), many circulating T cells and liver T cells in humans express NK receptors such as CD56 or CD161 (16, 17) and are not CD1d restricted or reactive to α-GalCer/CD1d (1).

Despite homogeneous Vα24/Vβ11 TCR segment expression or α-GalCer/CD1d specificity, human iNKT cells are phenotypically and functionally heterogeneous (18). Functional discrepancies have been attributed to iNKT cell subsets based on the expression of CD4 and CD8. All three human iNKT cell subsets, i.e., CD4$^+$, double negative (DN; CD4/CD8) and CD8$^+$, stimulated ex vivo with PMA/ionomycin, can produce Th1 cytokines (IFN-γ and TNF-α) or IL-2. Th2 cytokine production (IL-4, IL-10, and IL-13) is confined to the CD4$^+$ subset, whereas DN iNKT cells produce little or no IL-4 (9, 10, 19). Such differences between CD4$^+$ and

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[3] Abbreviations used in this paper: iNKT, invariant NKT cell; DAPI, 4′,4′-diamidino-2-phenylindole; DC, dendritic cell; DN, double negative (CD4/CD8); α-GalCer, α-galactosylceramide; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HD, healthy donor; IHL, intrahepatic lymphocyte; PBC, primary biliary cirrhosis; SP, single positive; TIL, tumor-infiltrating lymphocyte.

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$CD4^-$ iNKT cell subsets are less clear in the mouse and have been less intensively investigated (3). In addition, whereas both $CD4^+$ and $CD4^-$ (comprising DN and $CD8^+$) human iNKT cells can promote B cell proliferation, only $CD4^+$ iNKT cells can sustain Ab production in vitro (20). iNKT cell subsets also express different arrays of homing/chemokine and NK receptors (21, 22). With regard to tumor immunity, the $CD4^+$ subset has been shown to inhibit in vitro proliferation of tumor Ag-specific $CD8^+$ T cells through their production of IL-4 and IL-10 (23).

Mouse $CD4^+$- and CD1d-restricted $NK1.1^+$ or $DX5^+$ T cells can also suppress immune responses in vivo in sarcoma (24), skin cancer (25), or colon carcinoma (26). An additional study revealed that murine type II NKT cells (non-Vα14Jα18 invariant, non-α-GalCer reactive) are sufficient to promote tumor recurrence in these models (27). However, mouse iNKT cells have been implicated in the development of T lymphoma (28), and only the hepatic $CD4^-$ iNKT cell subset seems to perform α-GalCer-mediated protection against tumors (29). Therefore, it is possible that the distinct effects of iNKT cells in neoplastic disease may rely on different iNKT cell subpopulations (3). This has also been suggested in patients with asthma, where most $CD4^+$ T cells infiltrating the lung have been identified as $CD4^+V\alpha24^+$ iNKT cells producing IL-4 and IL-13 (30).

Various iNKT cell abnormalities have been reported in cancer patients. Peripheral iNKT cell frequencies were decreased in patients with prostate cancer (31), lung carcinoma (32), cutaneous melanoma (33), and breast cancer (34). iNKT cells derived from prostate cancer patients were deficient in IFN-γ release, this being associated with an increased IL-4/IFN-γ cytokine ratio in vitro (31). Although slightly decreased percentages of iNKT cells were found in PBL from myeloma patients, α-GalCer-induced IFN-γ production was detectable in both peripheral blood and the tumor bed of patients with nonprogressive myeloma, but not in progressive disease (35). Finally, circulating Vα24/Vβ11 iNKT cells from patients with glioma displayed similar frequency, CD4/CD8 phenotype distribution, and reactivity to α-GalCer, as compared with healthy donors (HD) (36).

Hepatic murine iNKT cells display potent antimetastatic effects in vivo upon specific activation with synthetic α-GalCer (37). These effects involve the ability of α-GalCer-stimulated iNKT cells to induce IL-12 secretion by myeloid cells presenting α-GalCer and to induce tumoricidal activity of NK cells (37). A physiological activity against early primary tumors have been attributed to iNKT cells in the absence of α-GalCer treatment by using $J\alpha18^{-/-}$ mice that selectively lack iNKT cells (38, 39). Interestingly, only the liver-derived DN subset was found to have antitumor activity (29). α-GalCer-stimulated human iNKT cells can also induce tumoricidal activity by NK cells in vitro (40, 41), and this might be important for the treatment of patients with intrahepatic malignancies. However, iNKT cells are found at lower levels in liver than their murine counterparts (8, 11, 42), with frequencies not significantly different from those observed in PBL (43). iNKT cells have been found to represent 0.06% of lymphocytes in both PBL and intrahepatic lymphocytes (IHL) of healthy donors (11). The frequency in the liver was increased to 0.2% in patients with primary biliary cirrhosis (PBC) and associated with an increased proportion of the $CD4^+$ iNKT cell subset (28 vs 49%) (11). A recent study reported that iNKT cell numbers were increased in the liver of patients with hepatitis C virus (HCV) cirrhosis (1.9% of $CD3^+$ cells) compared with patients with nonviral hepatic pathologies (benign and malignant lesions, 0.36%) (44). Another work reported a normal hepatic frequency of 0.48% of $CD3^+$ cells that was decreased to 0.098% in livers bearing colon metastases, with $CD4^+$ iNKT cells representing 15–20% of liver iNKT cells in both cases (45). However, the status of intratumoral iNKT cells was not documented.

Altogether, the phenotype and functionality of iNKT cells in cancer patients have been documented in PBL but were infrequently investigated in tumors and corresponding non-neoplastic tissue. This led to the observation of numerical and/or functional iNKT cell deficiencies in most studies. Because malignancies can develop in or metastasize to the human liver, it is important to understand why resident iNKT cells have not been sufficiently protective against these tumors. The status of human iNKT cells in invasive malignancies located in the liver has not been investigated to date. Therefore, we performed for the first time, a comprehensive ex vivo analysis of iNKT cell populations in PBL, IHL, and tumor-infiltrating lymphocytes (TIL) of patients with liver malignancies. We assessed the frequency of iNKT cells (defined as Vα24/Vβ11 T cells) and the representation of the $CD4^+$, DN, and $CD8^+$ subsets. These studies were complemented with a comparison of cytolytic activity and cytokine production between representative healthy donor-derived, $CD4^+$, and DN/$CD8^+$ iNKT cell clones upon α-GalCer presentation by $CD1d^+$ tumors.

Materials and Methods

Patients

Nine patients with primary hepatocellular carcinoma (HCC), four patients with colon carcinoma metastasis, and four patients with uveal melanoma metastasis underwent partial hepatectomies at our center (Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland) and were included in this study upon written informed consent. The study protocol was accepted by the ethical committee of the University of Lausanne and conformed to the 1975 declaration of Helsinki. Tumor type, age, gender, and pathological status of each patient are depicted in Table I.

Tissue sampling and lymphocyte extraction

Tissue samples were obtained from central portions of non-necrotic tumor nodules and from nontumoral liver taken distantly from tumor tissue. Ex vivo lymphocyte preparation was immediately performed with a protocol adapted from our previous work (46). Briefly, tissues were washed three times in medium, cut into small pieces with scissors, and digested for 1 h at 37°C in collagenase V (Sigma-Aldrich). Cells were then passed through a cell strainer and separated on a Percoll gradient as described in an earlier study (46). Variable amounts of cells were obtained, depending on the tumor type and the pathological status and amount of accessible tissue. High cellular viability (>98% viability at trypan blue staining) was obtained. Cells were cooled for 10 min on ice before freezing in medium with 10% DMSO and 40% FCS. In parallel, PBL were isolated by centrifugation with Ficoll and frozen in the same manner.

Abs and staining

The Abs Vα24-FITC and Vβ11-PE (clones C15 and C21, respectively; Immunotech), CD4-PeRCP, CD8α-allophycocyanin, CD3-allophycocyanin, CD8α-allophycocyanin-Cy7 (BD Biosciences Pharmingen), and CD4-ECD (Cyto-Stat/Coulter clone T4-ECD) and a vehicle (DMSO) or α-GalCer-loaded human CD1d tetramers were prepared as described in a previous study (47).

Phenotypic ex vivo staining was performed after thawing in 5% FCS-supplemented PBS and blocking the cells in the presence of human Igs (300 μg/ml Redimmune; ZLB Bioplasma). Cells were stained with Abs for 30 min on ice, washed before the addition of DAPI, and filtered before analysis or cell sorting on a FACSVantage, FACScan, or FACSCalibur machine. To ensure the reliability of FACS acquisition, settings were performed in the same manner with aliquots of a unique batch of PBL from a healthy donor. The correspondence between $V\alpha24^+/V\beta11^+$ cells and tetramer$^+$ cells was confirmed in an additional set of experiments using an LSR-II machine.

Statistical analysis was done using the one-tailed paired Wilcoxon test to compare cell populations between different locations (PBL vs IHL, PBL vs TIL, and IHL vs TIL), and the one-tailed unpaired Mann-Whitney *U* test was used to compare cell populations between healthy donors PBL and patients PBL.

Table I. *Patient characteristics*

Tumor	Patient Code	Age/Gender	Cirrhosis	Intrahepatic Tumor Nodule(s)	Histology
HCC	LAU 250	58/Male	Yes	Multiples	Moderate inflammatory infiltration, HCV infected
	LAU 682	62/Male	No	Multiples	Well differentiated, moderate inflammatory infiltration, hemochromatosis
	LAU 717	76/Female	Yes	Multiples	Well differentiated
	LAU 751	81/Male	Yes	1	Poorly to moderately differentiated, moderate inflammatory infiltration, HCV infected
	LAU 784	53/Male	Yes	1	Preneoplastic lesion, moderate inflammatory infiltration, HBV infected
	LAU 836	60/Male	No	1	Minimal inflammatory infiltration
	LAU 876	66/Male	Yes	1	Well differentiated, moderate inflammatory infiltration
	LAU 901	68/Male	Yes	Multiples	Poorly to well differentiated, moderate inflammatory infiltration
	LAU 931	73/Male	Yes	1	Moderately differentiated, moderate inflammatory infiltration, hemochromatosis
Uveal melanoma metastasis	LAU 361	49/Female	No	1	
	LAU 710	66/Male	No	Multiples	
	LAU 731	52/Female	No	Multiples	
	LAU 806	31/Female	No	Multiples	
Colon metastasis	LAU 687	71/Female	No	1	Moderate inflammatory infiltration
	LAU 722	64/Female	No	Multiples	Moderately differentiated, minimal inflammatory infiltration
	LAU 726	55/Female	No	1	Moderately differentiated, minimal inflammatory infiltration
	LAU 779	57/Female	No	Multiples	Moderately differentiated, minimal inflammatory infiltration

Sorting of iNKT cells for cloning and functional analysis

iNKT cells from PBL of healthy donors were isolated by sorting after staining with anti-CD3, anti-Vα24, and anti-Vβ11 Abs. The sorted cells were cloned by limiting dilution culture in the presence of 1 μg/ml PHA and 150 $\times$ 10^3 irradiated allogeneic PBL. Clones were maintained in culture by restimulation every 2–3 wk with PHA and irradiated allogeneic PBL, followed by medium exchange every 2 days.

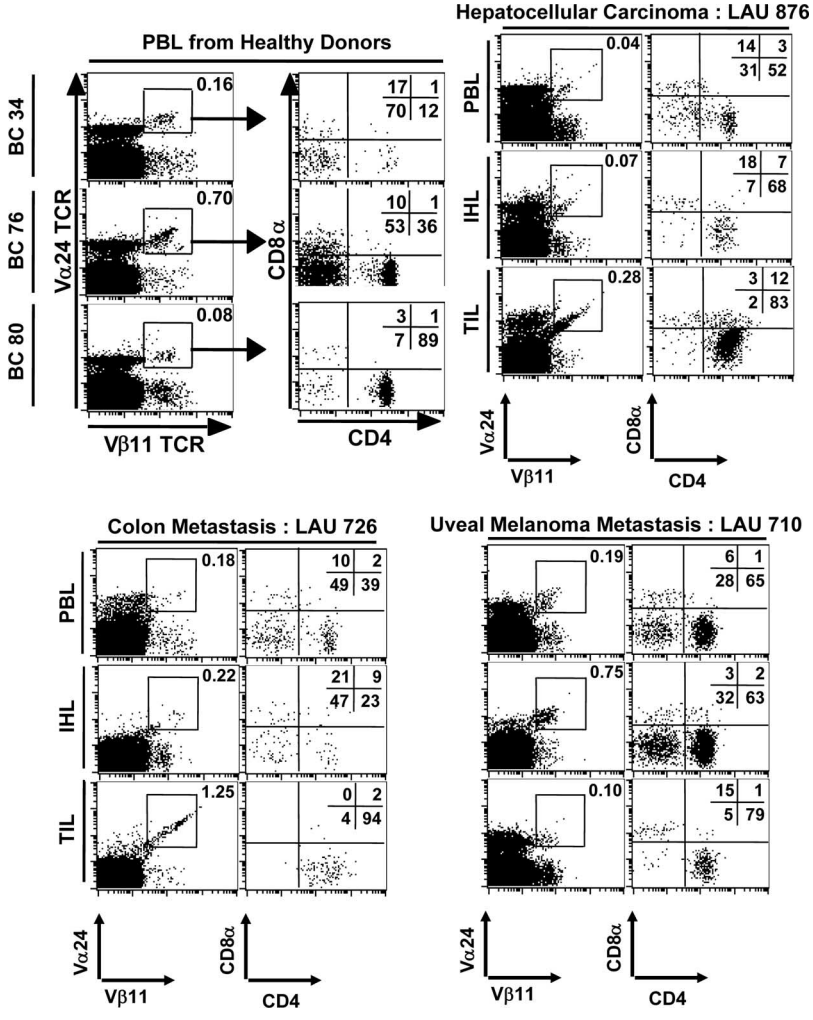

FIGURE 1. Flow cytometric analysis of Vα24/Vβ11 iNKT cell frequency and CD4/CD8α expression in blood of healthy donors and in blood, liver, and tumor of patients with tumor-invaded liver. Cells were extracted ex vivo, as described in *Materials and Methods*, by Ficoll centrifugation for PBL and tissue digestion and separation on a Percoll gradient for IHL and TIL. Flow cytometric analyses are shown for three HD (BC 34, BC 76, and BC 80) and representative patients with HCC (LAU 876), colon carcinoma metastasis (LAU 726), or uveal melanoma metastasis (LAU 710). Vα24 and Vβ11 FACS stainings are shown after gating on live T lymphocytes (CD3$^+$/DAPI$^-$ and FSC/SSC gated lymphocytes). Numbers for Vα24$^+$/Vβ11$^+$ cells indicate percentages among CD3$^+$ cells. The expression of CD4 and CD8α is shown for cells gated on Vα24$^+$/Vβ11$^+$ cells, with the percentages of cells indicated in the four quadrants.

Chromium release assay

The day before the experiment, 2×10^6 HeLa-CD1d transfected cells were placed in 25-cm^2 flasks with α-GalCer (KRN7000) at 100, 25, 6.2, 1.6, 0.4, or 0 ng/ml. As controls, mock-transfected HeLa cells were prepared in the same manner with and without α-GalCer at 100 ng/ml. In parallel, C1R-CD1d and C1R cells were prepared in a comparable manner and placed in 6-well plates. On the day of the experiment, cytotoxicity was assessed using target cells labeled with ^{51}Cr for 1 h at 37°C and washed three times in medium. Labeled target cells (3000 cells per well) were incubated with effector cells at an E:T cell ratio of 10:1 in V-bottom microwell plates and supernatants were collected after 4 h of incubation at 37°C.

Cytokine secretion assay

The day before the experiment, 25×10^3 HeLa-CD1d-transfected cells were placed in a flat-bottom, 96-well plate with α-GalCer at 100, 25, 6.2, 1.6, 0.4, or 0 ng/ml (final volume of 200 μl). As controls, mock-transfected HeLa cells were prepared in the same manner with and without α-GalCer at 100 ng/ml. C1R-CD1d transfected cells (2×10^6) were placed in 6-well plates with α-GalCer (KRN7000) at 100, 25, 6.2, 1.6, 0.4, or 0 ng/ml (final volume of 5 ml). As controls, mock-transfected C1R cells were prepared in the same manner with and without α-GalCer at 100 ng/ml.

The day of stimulation, plates with HeLa cells were washed twice with PBS before the addition of 50×10^3 iNKT cells per well. C1R cells were irradiated (10,000 rad) and placed at 50×10^3 cells per well in flat-bottom, 96-well plates together with of 50×10^3 iNKT cells per well. The level of cytokines released in supernatants, after 72 h of culture, was determined using a cytometric bead array (BD cytometric bead array human Th1/Th2 cytokine kit; BD Biosciences) conducted according to the manufacturer's guidelines. Detection limits of cytokine values ranged from 1 to 5000 pg/ml, and supernatants were diluted to fit in this range.

Results

Frequency of Vα24/Vβ11 iNKT cells in healthy donors and in patients with tumor-invaded liver

Seventeen patients bearing the most frequent intrahepatic malignancies were enrolled (Table I) and included patients with HCC ($n = 9$), colon carcinoma metastasis ($n = 4$), or uveal melanoma metastasis ($n = 4$). Paired specimens from both tumor and distant "normal" liver tissue were harvested. For comparison, peripheral blood samples from these patients and from 15 HD were also obtained.

First, we assessed individual and mean frequencies of iNKT cells in the blood of patients and HD by flow cytometry (Fig. 1). Importantly, a limited number of samples revealed that Vα24/Vβ11 T cell frequencies closely correlated with α-GalCer/CD1d tetramer$^+$ cell frequencies from the same sample (Fig. 2), thus validating the use of the combined Vα24/Vβ11 labeling to evaluate iNKT cell frequencies in clinical samples. This also suggests that α-GalCer/CD1d-reactive Vα24$^-$ or Vβ11$^-$ populations and noninvariant Vα24/Vβ11 T cells were undetectable or rare in our patient samples.

The frequency of circulating iNKT cells in the group of 15 HD was highly variable (Figs. 1 and 3*A* and data not shown). Circulating iNKT cell frequencies in cancer patients were reduced and less heterogeneous, but no statistically significant difference was found between peripheral iNKT cell frequencies from healthy donors and patients.

Next, we assessed iNKT cell frequencies in IHL and TIL (Figs. 1 and 3*A*). As compared with PBL, a significant increase in iNKT cell frequencies in TIL was observed when all 17 patients were considered ($p = 0.029$; Table II). In HCC patients, the frequency of iNKT cells in IHL was not elevated when compared with PBL (Fig. 3*A* and Table II). In contrast, the iNKT cell frequency in TIL was ~2-fold increased as compared with both PBL and IHL. Such increase was observed in most HCC patients and was statistically significant ($p = 0.0273$ and 0.0064, respectively). Patients with uveal melanoma metastases displayed increased iNKT cell frequencies in IHL and TIL as compared with PBL. However, these differences were not statistically significant ($p = 0.0625$ and 0.3125, respectively). The frequency of iNKT cells in IHL from patients with colon carcinoma metastasis was not increased compared with PBL, but the TIL did display a slight increase (Fig. 3*A* and Table II). However, these differences did not achieve statistical significance ($p = 0.50$ and 0.3125, respectively).

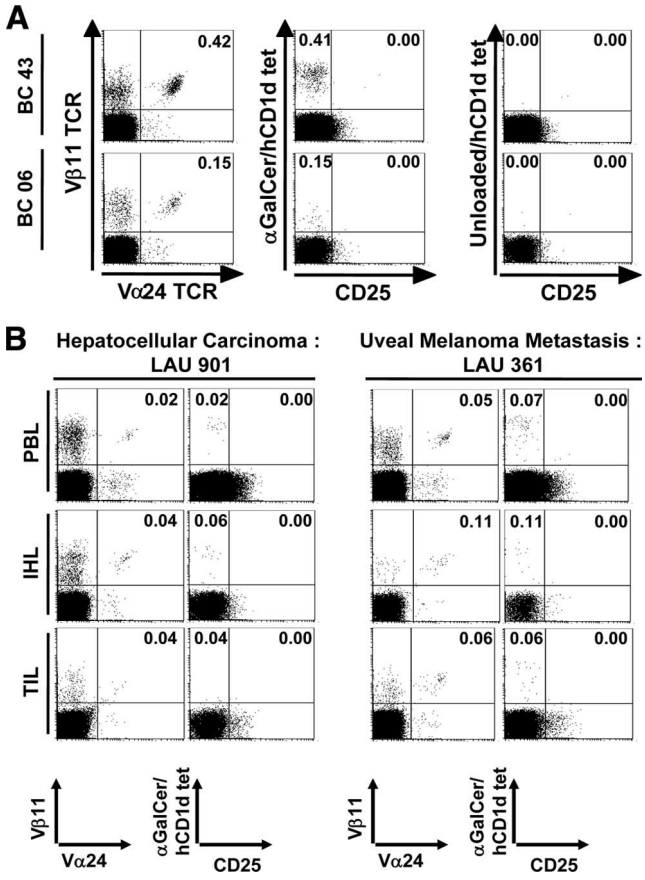

FIGURE 2. Correspondence between Vα24/Vβ11 cell frequency and α-GalCer/CD1d tetramer$^+$ cell frequency. Cells from the same samples were split into equal fractions to analyze in parallel Vα24$^+$/Vβ11$^+$ cells and α-GalCer/CD1d tetramer$^+$ cells. Flow cytometric stainings are shown after gating on live T lymphocytes (CD3$^+$/DAPI$^-$ and forward scatter/side scatter-gated lymphocytes). *A*, Analysis of PBL from two healthy donors (BC 6 and BC 43); *left column*, Vα24 and Vβ11 FACS staining are shown with percentages of Vα24$^+$/Vβ11$^+$ cells; *middle column*, α-GalCer/human CD1d tetramer (hCD1d tet) and CD25 staining with the percentages of tetramer$^+$/CD25$^-$ and tetramer$^+$/CD25$^+$ cells indicated; *right column*, control staining with vehicle (DMSO)-loaded tetramers. Data are representative for a total of five HD analyzed. *B*, Analysis of PBL, IHL, and TIL from patients with HCC (LAU 901) or uveal melanoma metastasis (LAU 361); *left column*, Vα24 and Vβ11 FACS staining; *right column*, α-GalCer/hCD1d tetramer and CD25 staining. Data are representative of two other HCC patients.

CD4$^+$, DN, and CD8α$^+$ subsets of Vα24/Vβ11 iNKT cells in healthy donors and patients with tumor-invaded liver

The three iNKT cell subsets (CD4$^+$, DN, and CD8$^+$) were present in all samples analyzed, and a fourth iNKT cell subset that is CD4/CD8 double positive was also detected in some samples (Fig. 1). Because members of this fourth subset were CD4$^+$ and only weakly represented, they were included in the CD4$^+$ subset for subsequent analysis.

Again, we found large subset variations in HD (Fig. 3*B* and Table II). On average, 44 ± 24% of NKT cells were CD4$^+$ and

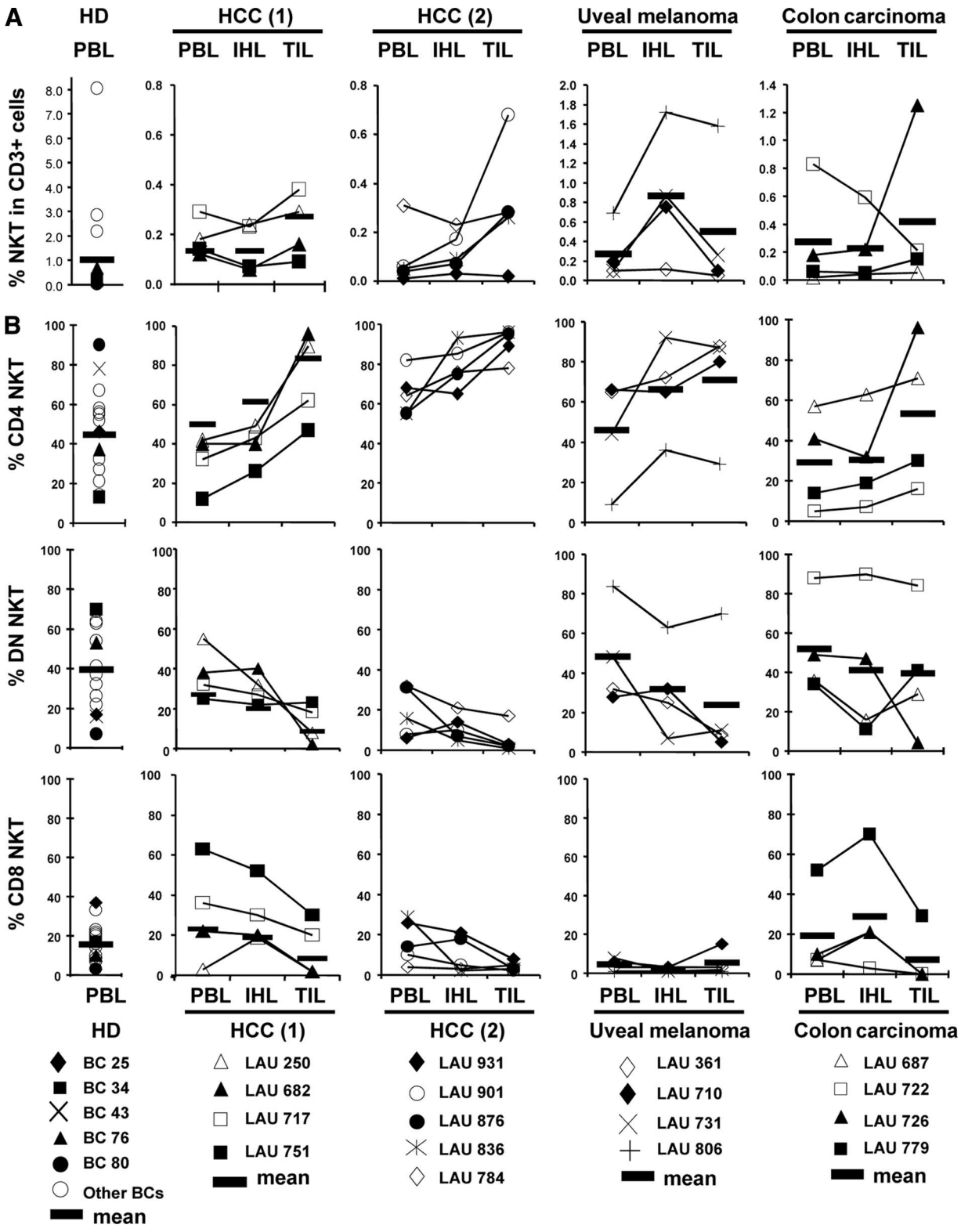

FIGURE 3. Vα24/Vβ11 iNKT cell frequencies and CD4$^+$/DN/CD8α^+ subsets in blood of healthy donors and in blood, liver and tumor of patients with tumor-invaded liver. *A*, Individual and mean frequencies of Vα24$^+$/Vβ11$^+$ iNKT cells among live T cells in PBL from HD (n = 15) and PBL/IHL/TIL from patients with HCC (n = 9), uveal melanoma (n = 4), or colon carcinoma (n = 4) liver metastasis. For better visualization of HCC patients, the data were split into two series, HCC (1) and HCC (2). Mean values for each series are represented as horizontal thick bars. Each donor is represented by a symbol (see legend at the bottom of the figure). *B*, Individual and mean frequencies of CD4$^+$ (CD4$^+$/CD8α^- single positive and CD4$^+$/CD8α^+ double positive cells)-, DN (CD4$^-$/CD8$^-$)-, and CD8$^+$ (CD4$^-$/CD8$^+$)-expressing cells among Vα24$^+$/Vβ11$^+$ iNKT cells. Individual and mean data are presented in the same manner as in *A*.

39.5 ± 21% were DN. CD8$^+$ iNKT cells were consistently underrepresented, accounting for 16.5 ± 8% of total iNKT cells. In PBL from patients with HCC and uveal melanoma, the CD4$^+$ iNKT cell subset was of a similar size (Fig. 3*B* and Table II), and varied between patients. Lower values were observed in colon carcinoma patients. A significant decrease was observed in peripheral DN

Table II. *iNKT cell frequencies and iNKT subset distributions (mean ± SD)*

	iNKT in $CD3^+$ Cells (%)	$CD4^+$ in iNKT (%)	DN in iNKT (%)	$CD8^+$ in iNKT (%)
Healthy donors				
PBL	1.00 ± 2.13	44.0 ± 24.1	39.5 ± 21.2	16.5 ± 9.8
HCC				
PBL	0.133 ± 0.109[a]	50.0 ± 21.0[a,b]	27.0 ± 15.4[a,b,c]	23.0 ± 18.8[a]
IHL	0.132 ± 0.085[a]	61.3 ± 22.8[a,d]	19.8 ± 11.9[a,d]	18.9 ± 15.6[a]
TIL	0.271 ± 0.190[b,d]	83.2 ± 17.7[b,d]	8.4 ± 8.6[b,d]	8.3 ± 10.0[b,d]
Uveal melanoma metastasis				
PBL	0.270 ± 0.283	46.0 ± 26.7	48.0 ± 25.5[a]	4.5 ± 3.1[c]
IHL	0.860 ± 0.662	66.3 ± 23.2	31.8 ± 23.3	2.0 ± 1.2
TIL	0.498 ± 0.727	71.0 ± 28.2	23.8 ± 30.9[d]	5.3 ± 6.6
Colon carcinoma metastasis				
PBL	0.273 ± 0.378	29.3 ± 24.0	51.8 ± 25.1	19.0 ± 22.0[a]
IHL	0.225 ± 0.257	30.3 ± 24.1	41.0 ± 36.3	28.8 ± 28.8[a]
TIL	0.415 ± 0.561	53.3 ± 36.8	39.5 ± 33.4	7.3 ± 14.5[b,d]
All patients together				
PBL	0.198 ± 0.230[a]	44.2 ± 23.2[a,b]	37.8 ± 22.3[a,b]	17.7 ± 18.1[a]
IHL	0.325 ± 0.439	55.2 ± 26.0[a,d]	27.6 ± 22.4[a,d]	17.2 ± 19.3[a]
TIL	0.358 ± 0.431[d]	73.3 ± 26.8[b,d]	19.4 ± 24.5[b,d]	7.4 ± 9.9[b,d]

[a] Statistically different from the population in TIL.
[b] Statistically different from the population in IHL.
[c] Statistically different from the population in HD PBL.
[d] Statistically different from the population in patient PBL.

iNKT cells from HCC patients. Whereas both HCC and colon carcinoma patients had normal frequencies of $CD8^+$ iNKT cell subsets, the frequencies were decreased in patients with uveal melanoma (Table II).

Altogether, in PBL we found almost no significant differences in the distribution of $CD4^+$, DN, and $CD8^+$ iNKT cells from HD and patients. The only exception was a decrease in DN iNKT cells in HCC patients ($p = 0.0136$) and a consistent lack of $CD8^+$ iNKT cells in PBL from uveal melanoma patients as compared with HD ($p = 0.0081$). By contrast, the iNKT subset distribution in the liver was different. Indeed, the analysis of the iNKT cell repertoire according to CD4 and CD8 expression between blood and hepatic and tumoral compartments in patients revealed interesting differences:

In HCC patients, we found a consistent increase in $CD4^+$ iNKT cells between PBL, IHL, and TIL (Fig. 3*B* and Table II). This increase was statistically significant from PBL to IHL ($p = 0.0124$), from PBL to TIL ($p = 0.0045$), and from IHL to TIL ($p = 0.002$). Both HCC patients with low/normal $CD4^+$ iNKT cell fractions in PBL (HCC series 1; Fig. 3*B*) and those with elevated $CD4^+$ iNKT cell fractions (HCC series 2; Fig. 3*B*) displayed increased $CD4^+$ iNKT cell fractions in both IHL and TIL. This coincided with diminished DN and $CD8^+$ fractions among iNKT cells in IHL and even more so in TIL. DN iNKT cells, already represented less than in HD PBL, decreased among PBL, IHL, and TIL (Fig. 3*B*). This decrease was statistically significant from PBL to IHL ($p = 0.0483$), from PBL to TIL ($p = 0.0045$), and from IHL to TIL ($p = 0.0064$). $CD8^+$ iNKT cells were also less represented in TIL as compared with PBL or IHL. Again, this decrease was statistically significant from PBL to TIL ($p = 0.0075$) and from IHL to TIL ($p = 0.0098$). Moreover, patients LAU 751 and LAU 717, who showed a high $CD8^+$ iNKT cell fraction in PBL compared with HD, also had decreased $CD8^+$ iNKT numbers in IHL and even less in TIL (Fig. 3*B*).

In metastases of patients with uveal melanoma, a similar trend for the enrichment for $CD4^+$ iNKT cells and the loss of other iNKT cell subsets was found. The mean fraction of $CD4^+$ iNKT cells increased from PBL to IHL to TIL (Fig. 3*B* and Table II). In addition, the mean fraction of DN iNKT cells decreased from PBL to IHL or TIL and was significantly different between PBL and TIL ($p = 0.0488$). Interestingly, $CD8^+$ iNKT cells were rare to absent in all compartments analyzed (Fig. 3*B* and Table II).

In patients with colon carcinoma metastases, iNKT cell subsets were similarly represented between PBL and IHL. However, TIL displayed an increased proportion of $CD4^+$ iNKT cells (Fig. 3*B*). DN iNKT cells were more represented in PBL than in IHL or in TIL. $CD8^+$ iNKT cells were more highly represented in IHL than in PBL. On the contrary, a significant loss of $CD8^+$ iNKT was observed in tumors (from PBL to TIL, $p = 0.0048$; from IHL to TIL, $p = 0.0048$).

Within samples of uveal melanoma and colon carcinoma, the differences in CD4/CD8 iNKT cell subsets were less statistically significant despite the observed trends. This may be due to the low numbers of patients analyzed for each tumor type ($n = 4$) and to heterogeneity between patients. However, when compiling the data of all 17 patients (Fig. 4), the differences were statistically significant.

$CD4^+$, DN, and $CD8^+$ Vα24/Vβ11 iNKT cells subsets vary independently of total $CD3^+$ T cells

We investigated whether the described distribution of iNKT cell subsets correlated with CD4/CD8 ratio shifts in other T cell populations. Thus, we analyzed the proportions of $CD4^+$, DN, and $CD8^+$ cells among iNKT cells in relation to total $CD3^+$ T cells, as well as Vα24 single positive (SP) cells ($V\alpha24^+V\beta11^-$) and Vβ11 SP cells ($V\beta11^+V\alpha24^-$) (Fig. 4). In circulating blood, no significant difference was observed between HD and patients both for iNKT cells and other T cell populations (Figs. 3*B* and 4). For iNKT cells in liver tissues from patients there was a progressive and significant increase of $CD4^+$ iNKT cell proportions between PBL and IHL or IHL and TIL ($p = 0.0026$ and 0.0005, respectively, and $p = 0.0002$ between PBL and TIL; Fig. 4). The DN iNKT cell subset appeared to be progressively decreased from PBL to IHL and TIL ($p = 0.0056$ and 0.0324, respectively, and $p = 0.0004$ between

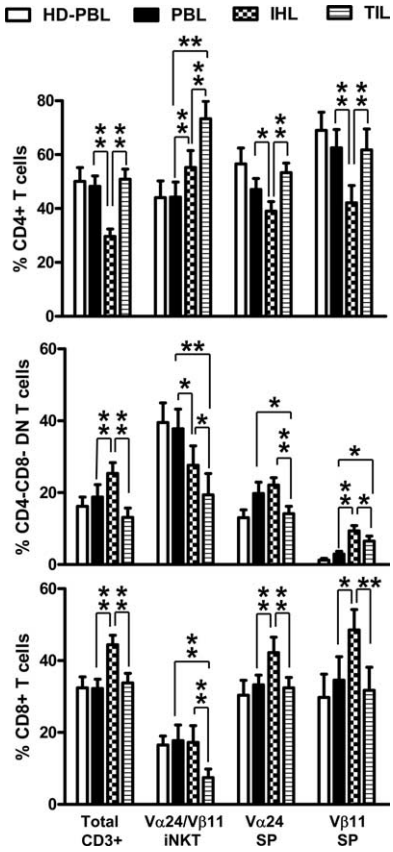

FIGURE 4. Comparison of CD4$^+$, DN, and CD8$^+$ subsets between total T cells, Vα24/Vβ11 iNKT cells, Vα24 single positive T cells, and Vβ11 single positive T cells in the blood of healthy donors and in the blood, liver, and tumor of patients with tumor-invaded liver. Mean frequencies of CD4$^+$, DN (CD4$^-$/CD8$^-$), and CD8α^+ subsets (*top*, *middle*, and *bottom panels*, respectively) among total CD3$^+$ T cells, Vα24$^+$/Vβ11$^+$ iNKT cells, Vα24 single positive T cells (Vα24 SP), and Vβ11 single positive T cells (Vβ11 SP) are shown. Values for PBL of HD (HD-PBL, open bars; $n = 15$) and for patient PBL (filled bars), IHL (checkered bars), and TIL (dashed bars; $n = 17$). SD values are indicated by brackets. The one-tailed paired Wilcoxon test was used to compare cell populations between different patient locations (PBL against IHL, PBL against TIL, and IHL against TIL). The one-tailed unpaired Mann-Whitney *U* test was used to compare cell populations between PBL of HD and PBL of patients. Values of $p < 0.05$ are indicated with asterisks: *, $p < 0.05$; **, $p < 0.005$.

PBL and TIL). The frequencies of CD8$^+$ iNKT cells were comparable between PBL and IHL but significantly lowered in TIL compared with PBL or IHL ($p = 0.0019$ and 0.0041, respectively).

For total CD3$^+$ T cells as well as Vα24 SP and Vβ11 SP, the variations in CD4$^+$, DN, and CD8$^+$ subsets in tissues were different from iNKT cells variations (Fig. 4). The variations among Vα24 SP and Vβ11 SP populations were similar to the variations of total CD3$^+$ cells. All patients displayed comparably high CD4$^+$ proportions and low CD8$^+$ proportions among CD3$^+$ cells from both PBL and TIL as compared with IHL. Populations in liver corresponded to the normal biology of liver (16), as CD8$^+$ T cells were the most represented in IHL and significantly increased compared with PBL and TIL. DN T cell frequencies were also significantly increased in the liver (Fig. 4). Altogether, the observed variations in the CD4/CD8 repertoire of iNKT cells were clearly independent of the percentages of CD4$^+$, DN, and CD8$^+$ cells in other T cell compartments.

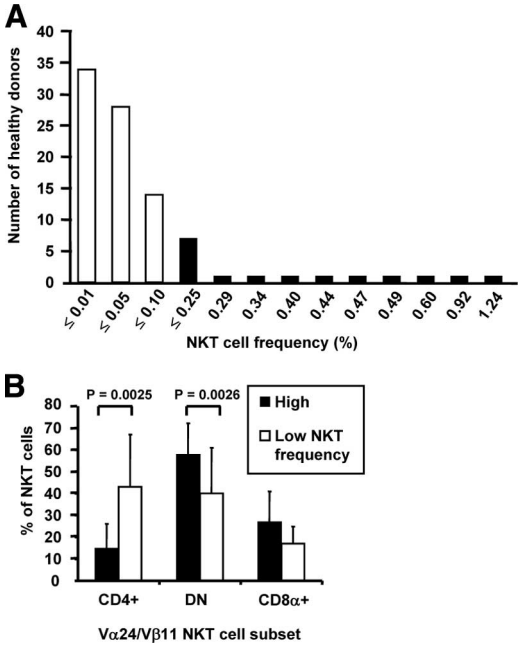

FIGURE 5. Circulating iNKT cells in healthy donors: Distribution classified according to frequency and enrichment of DN and CD8$^+$ iNKT cell subsets in donors with relatively high iNKT cell frequency. *A*, PBL from 92 HD were stained ex vivo with fluorescent anti-Vα24 and anti-Vβ11 mAbs and analyzed by flow cytometry. The frequency of Vα24/Vβ11 iNKT cells was assessed after gating on lymphocytes. Donors with frequencies $\leq$0.25% were grouped into four classes. The average frequency among all donors was 0.09 $\pm$ 0.18% and donors with a frequency $> 0.25\%$ are depicted by filled bars whereas donors with a frequency $\leq 0.25\%$ are depicted by open bars. *B*, Percentages of CD4$^+$, DN, and CD8$^+$ subsets among gated iNKT cells. PBL from HD with relatively low (open bars; $n = 10$) or high (filled bars; $n = 10$) iNKT cell frequencies were stained ex vivo with fluorescent anti-Vα24, anti-Vβ11 mAbs, anti-CD4, and anti-CD8α mAbs before analysis by flow cytometry. The one-tailed unpaired Mann-Whitney *U* test was used to analyze statistical differences between populations; values of $p < 0.05$ are indicated.

Enrichment of CD4$^-$ Vα24Vβ11 iNKT cell subsets in the blood of healthy donors with relatively high NKT cell frequencies

We had initially observed (data not shown) that HD with the highest iNKT cell frequencies were enriched in CD4$^-$ iNKT cells. Therefore, we assessed iNKT cell frequencies in a larger cohort of HD ($n = 92$). The iNKT cell frequency among gated lymphocytes ranged from the detection limit (0.01%) to 1.24%, with a mean frequency of 0.09 $\pm$ 0.18%. When we ranked donors according to their respective iNKT cell frequency (Fig. 5*A*), they could be classified into two groups. The majority presented ($n = 76$; ~83% of normal individuals) with low percentages close to the detection limit (0.03 $\pm$ 0.03% on average), and a minority ($n = 16$) with high percentages (0.37 $\pm$ 0.3%). We then compared CD4$^+$, DN and CD8$^+$ iNKT cell subsets between HD with relatively low or high iNKT cell frequencies (Fig. 5*B*). We observed that HD with high iNKT cell levels displayed a significantly decreased proportion of CD4$^+$ iNKT cells compared with HD with low iNKT cell levels; this resulted in significantly increased percentages of DN iNKT cells. The proportions of CD8$^+$ iNKT cells were also increased but were not statistically significant.

We concluded that HD with relatively high iNKT cell frequencies displayed a decreased proportion of their CD4$^+$ subset together with an increase in the CD4$^-$ subsets. This contrasts sharply with the enrichment of CD4$^+$ iNKT cells inside the tumors of patients.

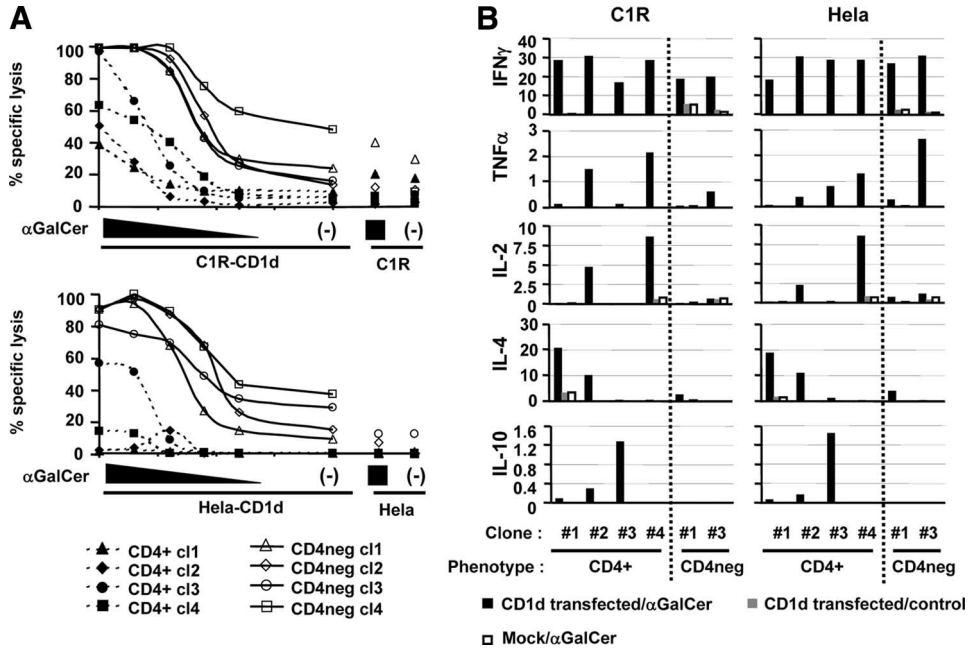

FIGURE 6. Distinct functional responses of $CD4^-$ and $CD4^+$ Vα24Vβ11 iNKT cells to $CD1d^+$ tumor cells. $CD4^-$ and $CD4^+$ iNKT cells clones were generated from HD by limiting dilution of flow cytometry-sorted V$\alpha24^+$/V$\beta11^+$/$CD3^+$ cells and expansion by PHA stimulation. *A*, Cytolytic activity of $CD4^+$ clones (cl1, cl2, cl3, and cl4) (filled symbols) and $CD4^-$ (CD4neg) clones (open symbols) against C1R (*upper panel*) and HeLa cells (*lower panel*). The activity was assessed against CD1d-transfected targets pulsed overnight with 100, 25, 6.2, 1.6, 0.4, or 0 ng/ml of α-GalCer. The cytolytic activity was also assessed against mock-transfected cells pulsed overnight with 100 or 0 ng/ml α-GalCer. The CTL activity was tested in 4-h chromium release assays at a lymphocyte to target cell ratio of 10:1. *B*, Cytokine release of $CD4^+$ and $CD4^-$ iNKT cell clones stimulated with C1R and HeLa cells. The cytokine release was assessed using CD1d-transfected cells pulsed overnight with 100 ng/ml α-GalCer (filled bars) or pulsed with control vehicle (DMSO; gray bars) and mock-transfected cells pulsed with α-GalCer (white bars). After 72 h of culture, supernatants were recovered and cytokine levels were assessed using a human Th1/Th2 cytokine bead array kit for the simultaneous detection of IFN-γ, TNF-α, IL-2, IL-4, and IL-10. Cytokine concentrations are expressed in ng/ml.

$CD4^-$ and $CD4^+$ Vα24Vβ11 iNKT cell subsets display functionally different responses to $CD1d^+$ tumor cells

Functional specialization among $CD4^+$ and $CD4^-$ human iNKT cells has been suggested previously (9, 10, 19). In addition, adoptive transfer studies in mice recently reported that liver-derived $CD4^+$ iNKT cells were weakly protective against tumors compared with their $CD4^-$ counterparts (29).

To functionally characterize iNKT cells derived from patients, PBL, IHL, and TIL from the 17 patients were systematically sorted during flow cytometry analysis. Sorted iNKT cells proliferated poorly, despite stimulation with PHA and IL-2 (data not shown). In sharp contrast, iNKT cells sorted from the blood of HD expanded vigorously when cultured in the same conditions (data not shown). In addition, iNKT cells derived from patient samples and detected with tetramers did not express the activation marker CD25, neither in blood nor in IHL and TIL (Fig. 2). Because we could not culture iNKT cells from patients, we generated $CD4^+$ and $CD4^-$ iNKT cell clones from HD and performed functional analysis (Fig. 6).

In cytolytic assays using CD1d-transfected C1R and HeLa target cells, $CD4^-$ iNKT clones displayed higher cytolytic activity when compared with their $CD4^+$ counterparts (Fig. 6*A*). $CD4^-$ iNKT clones all showed higher cytolytic activity induced by the highest Ag concentration and required lower Ag concentrations to achieve half-maximal lysis (EC_{50} ranged from 1 to 3.4 ng/ml α-GalCer for $CD4^-$ clones and from 3.1 to 22.8 ng/ml α-GalCer for $CD4^+$ clones).

In cytokine secretion assays using the same APCs, both $CD4^+$ and $CD4^-$ clones displayed the ability to secrete comparable amounts of IFN-γ and variable amounts of TNF-α (Fig. 6*B*). $CD4^-$ NKT cell clones produced only little or no IL-2, IL-4, and IL-10, whereas their $CD4^+$ counterparts produced relatively high amounts of these cytokines (Fig. 6*B*). Intriguingly, $CD4^+$ clones behaved differently, with one clone producing IL-2, IL-4, and IL-10 (no. 2), and others producing either IL-4 (no. 1), IL-10 (no. 3), or IL-2 (no. 4) alone.

Altogether, in response to $CD1d^+$ tumor cells, $CD4^-$ iNKT cell clones displayed higher CTL activity and produced lower levels of Th2 cytokines and IL-2, compared with $CD4^+$ clones. Similar results were obtained with $CD4^+$ and $CD4^-$ polyclonal iNKT cell lines (data not shown).

Discussion

We studied human iNKT cells ex vivo to determine their repertoire in blood and hepatic and tumoral compartments, excluding variations in $CD4^+$, DN, and $CD8^+$ iNKT cell subsets that may arise during in vitro expansion with mitogens or α-GalCer (11). However, liver samples from healthy individuals were not available as a physiological control of normal IHL.

iNKT cell levels in PBL were reduced, on average, in cancer patients compared with PBL from HD. This decrease was not statistically significant, in part because iNKT cell levels in patient PBL appeared similar to those in the majority of HD with low levels.

A central finding was that total iNKT cell frequencies were significantly higher in tumors as compared with circulating blood, which was most evident in HCC patients.

The mean iNKT cell frequency in livers from all cancer patients was in the same range of frequencies reported in normal liver or liver with primary biliary cirrhosis (11, 45). However, we observed

some disparities depending on the tumor type, with higher iNKT cell frequencies in IHL of patients with uveal melanoma compared with those with HCC (Table II). The mean iNKT cell frequency that we found in IHL of four patients with colon metastases was higher than the one reported previously (0.225 vs 0.098% among CD3$^+$ cells) (45). We did not observe a decrease of total iNKT cells, but two of four patients did, however, display a lower frequency. Studies with larger patient numbers are required for more definitive results.

It has been reported that HCV patients with active cirrhosis displayed increased iNKT cell frequencies in liver (44). Our data, generated from both liver and tumor of HCC patients (including patients LAU 784 and LAU 250/LAU 751 with HBV and HCV, respectively), did not show such a dramatic increase, suggesting that iNKT cell homeostasis is dysregulated in the case of chronic liver infection but nearly not in the case of established intrahepatic tumors. The iNKT cell frequency in liver of patients with HCC indeed did appear lower than that in other patients and might be related to the (mostly nonviral) cirrhosis context.

The highest mean iNKT cell frequency in IHL was observed in patients with uveal melanoma metastases and was nearly significantly increased compared with the mean levels in PBL. Three of four patients displayed a frequency of at least 0.7%, suggesting that iNKT cells levels in nontumoral liver might be specifically increased in the context of uveal melanoma metastasis. Again, larger patient numbers are required to confirm these hypotheses.

Most patients displayed similar iNKT subset distribution in peripheral blood compared with HD with even less variation, suggesting that in the types of tumors investigated in this study the CD4/CD8 phenotypes of iNKT cells in patient blood was not skewed. This is supported by comparable observations reported for patients with glioma (36) and breast carcinoma (48). Nevertheless, this was not the case for DN iNKT cells in HCC patients, and a significant lack of CD8$^+$ iNKT cells was found in all patients with uveal melanoma. Interestingly, a decrease of circulating CD8$^+$ iNKT cells has been observed previously in patients with chronic HCV infection (49). On the contrary, we (Fig. 5) and others (50) observed increased proportions of both DN and CD8$^+$ iNKT cells in HD with relatively high iNKT cell frequencies; it has been suggested that this occurs transiently in response to benign infections (50). However, additional analyses are required to determine why this does not occur in the case of persistent diseases such as cancer or HCV infection.

The distribution of CD4$^+$, DN, and CD8$^+$ iNKT cell subsets has been previously documented in livers from healthy subjects and patients with PBC or colon carcinoma metastasis. Fifteen to 28% of iNKT cells from normal liver were CD4$^+$ (11, 45), which was always less than or equal to the proportion in blood. The distribution of iNKT cell subsets in livers bearing colon carcinoma metastases has been investigated previously and revealed that ~20% of iNKT cells were CD4$^+$ (45). We obtained a comparable value in our patients, with 30% on an average for IHL. The variations in the CD4$^+$ subset could not be identified in the IHL of patients with colon carcinoma. However, the frequency of CD4$^+$ iNKT cells that we found in the livers of patients with HCC or uveal melanoma metastases were elevated compared with the frequencies reported in normal livers (61.3 or 66.3% against 15–28% of iNKT cells) (11, 45). In a comparable manner, the CD4$^+$ iNKT cell subset increased to 50% in livers with PBC (11). The dominance of CD4$^+$ iNKT cells inside tumor tissues (73.3% on average) was even more striking and suggests a progressive enrichment in CD4$^+$ iNKT cells from blood to liver to tumor. The fact that we made this observation in all of the three neoplastic diseases studied suggests that it occurred in vivo, independently of the tumor type or etiology of HCC. Additional analyses are required to determine whether this phenomenon is restricted to the liver or whether a similar enrichment may also occur in tumors located elsewhere. This trend was independent of total T cells, Vα24 SP cells, or Vβ11 SP T cells, as these were predominantly CD8$^+$ or DN in liver and predominantly CD4$^+$ in tumor. Thus, our observations are in agreement with a previous report concerning the normal biology of liver T cells (16) with a dominance of CD8$^+$ T cells, thus confirming the validity of our method of ex vivo lymphocyte isolation. The predominance of total CD4$^+$ T cells among tumors was previously described by flow cytometry and/or IHC in patients with HCC (46, 51) or colon metastases (52).

In functional assays with healthy donor-derived iNKT clones, the CD4$^+$ subset showed weak cytolytic activity but readily released IL-2, IL-4, and/or IL-10 in response to CD1d$^+$ tumor cells. Compared with the CD4$^-$ subsets, which displayed strong cytolytic activity and a Th1 cytokine profile, the CD4$^+$ subset may therefore be inappropriate for protection against tumor growth. In addition, the production of IL4/IL-10 by CD4$^+$ iNKT cells was found to inhibit the expansion of tumor Ag-specific CD8$^+$ T cells (23). Although a previous study showed human CD4$^+$ iNKT cells with higher cytolytic activity than DN iNKT cells, these results were generated with different APC and independently of CD1d presentation (53).

It has been recently reported that the DN iNKT cell subset in mouse liver, but not the CD4$^+$ one, shows α-GalCer-mediated antitumor activity (29). This notion fits with our data in the sense that CD4$^+$ iNKT cells may lack antitumor functions and may even favor tumor development. However, iNKT cell functional responses remain to be investigated in response to tumor-related glycolipid Ag(s), such as GD3 (54), and relevant CD1d$^+$ cells, which are resident in the liver and the tumor microenvironment.

We did not assess the expression of CD1d in our patient samples; the literature suggests that relevant tumor cells may express CD1d. CD1d is physiologically expressed by intestinal epithelial cells (55, 56), and some colon carcinoma cell lines, such as T84, CaCO2, Cl.19A, and HT29, express sufficient levels of CD1d for the presentation of α-GalCer to iNKT cells (57). Variable levels of CD1d expression in normal hepatocytes have been reported that range from weak (44, 58) to high levels (56), depending on the study. Interestingly, CD1d expression was up-regulated in the context of viral cirrhosis (44, 58). The HCC cell lines WIF-B9 (58) and HepG2 cells (59) seem to express low levels of CD1d. The expression of CD1d among normal melanocytes has not been documented, but CD1d could not be detected in the two cutaneous melanoma cell lines M-14 (41) and FO1 (60). It is thus likely that colon carcinoma and HCC cells might express CD1d for presentation to iNKT cells, but this might not be the case for uveal melanoma cells. Furthermore, CD1d presentation remains possible in the liver by resident dendritic cells (DC), Kupffer cells, Ito cells (61), and hepatocytes under inflammatory conditions (44, 58) or by B cell infiltrates detected by FACS in both IHL and TIL (data not shown).

The enrichment of CD4$^+$ iNKT cells in tumors might be promoted by a faster in situ proliferation of this subset. However, during our studies we noticed a proliferation defect in all iNKT cells from cancer patients upon PHA stimulation with IL-2 (not shown) that was not the case for iNKT cells from healthy donors. Such an impaired proliferative response has been previously reported in patients but could be overcome with G-CSF treatment (62). Functional defects of iNKT cells have also been reported in the context of established tumors in both patients and mouse models (31, 35, 63). The lack of CD25 expression by iNKT cells in our

patients further suggests that these cells may have functional defects.

It is also possible that $CD4^+$ iNKT cells have a preferential expression of homing receptors to infiltrate the tumor. The known chemokine receptors expressed by T cells infiltrating HCC or colon carcinoma are CCR5, CXCR3, and CXCR6 (64, 65). The CCR5 and CXCR3 receptors are commonly expressed by all iNKT cell subsets, and CXCR6 and CCR6 are preferentially expressed by the $CD4^-$ subsets (18, 22), suggesting that DN/$CD8^+$ iNKT cells display an enhanced ability to home to the liver and tumors generating CCR6 ligands. These facts probably suggest that there may be selective deletion of $CD4^-$ iNKT cells in liver and tumor tissue, because we observed decreases in DN or $CD8^+$ iNKT cell subsets in patients with HCC or uveal melanoma.

Human iNKT cells arise in the thymus as $CD4^+$ cells (66, 67), and the majority of iNKT cells in cord blood remain $CD4^+$ (66). These findings suggest that DN and $CD8^+$ iNKT cells may represent an expanded and further differentiated stage of iNKT cells. It is therefore of interest to know whether they are lacking because they were more prone to activation-induced cell death than their $CD4^+$ counterparts in vivo or whether differentiation from $CD4^+$ to $CD4^-$ iNKT cells was blocked in the tumoral context. A similar defect has been observed in the differentiation of melanoma-infiltrating, tumor Ag-specific $CD8^+$ T cells (68).

Four clinical trials using either free α-GalCer- or immature or mature α-GalCer-pulsed DC have been performed in patients with tumors of various histological origins (69–72). Some patients, who had significant circulating iNKT cell frequencies before therapy displayed detectable cytokines in the serum (69–71) and activation of bystander T, B and NK cells (70). Adoptive transfer of mature α-GalCer-pulsed DC increased iNKT cell numbers in the patient's PBL, with $CD4^+$ iNKT cells appearing after the first DC infusion and shifting toward dominance of $CD4^-$ after the second DC infusion (71). Unfortunately, no tumoral regressions were observed (69–72).

The weak improvement of disease through α-GalCer treatment may be explained by the low intrahepatic iNKT cell frequencies compared with those of mice and the inherent $CD4^+$ bias of tumor-infiltrating iNKT cells compared with blood or normal healthy liver (11, 45). Restoration of DN and $CD8^+$ iNKT cells, which are lacking in established intrahepatic tumors, may be beneficial when considering the clinical outcome. Adoptive transfer of DN/$CD8^+$ iNKT cells (73) or α-GalCer-pulsed mature DC to stimulate their expansion in vivo (71) might be particularly effective in patients with cancer located in the liver. Significant homing to the liver has been observed for i.v. transferred tumor Ag-specific $CD8^+$ T cells or α-GalCer-pulsed immature DC (70, 74), but further research is required to improve iNKT cell persistence after infiltration into these sites.

It is currently a topic of major interest to harness the adjuvant activity of iNKT cells to initiate innate and adaptive antitumor responses (75). Importantly, iNKT cell-based therapeutic protocols should focus on avoiding Th2 cytokine production from $CD4^+$ iNKT cells by using α-GalCer analogues that selectively induce Th1 cytokine production. An interesting candidate is the C-glycoside analog (α-C-GalCer) that demonstrated in mouse a superior antitumor activity as compared with α-GalCer and did not induce IL-4 in serum (76).

In conclusion, we have shown that the iNKT cell repertoire is influenced by neoplastic disease, which primarily affects tumor-infiltrating iNKT cells. Although the data presented here are from a limited number of patients, we hope that our study will stimulate more detailed research on human iNKT cells associated with tumors. Our observation is of major importance for understanding the biology of human iNKT cells. Recently, the majority of lung-infiltrating T cells in patients with asthma were identified to be invariant $CD4^+$ iNKT cells. They might contribute to this disease through their potent IL-4 and IL-13 release as demonstrated by ex vivo stimulation with PMA/ionomycin (30). Thus, the representation and functional differences between iNKT cell subsets, defined by their CD4/CD8 expression, appears to be important for the fate of immune responses. This should be the focus of more detailed investigation, particularly at disease sites, i.e., inside human tumors of various origins.

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Disclosures

The authors have no financial conflict of interest.

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