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Distinct subpopulations of γδ T cells are present in normal and tumor-bearing human liver

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Abstract

γδ T cells are thought to mediate immune responses at epithelial surfaces. We have quantified and characterized hepatic and peripheral blood γδ T cells from 11 normal and 13 unresolved tumor-bearing human liver specimens. γδ T cells are enriched in normal liver (6.6% of T cells) relative to matched blood (0.9%; $P = 0.008$). The majority express $CD4^{-}CD8^{-}$ phenotypes and many express CD56 and/or CD161. In vitro, hepatic γδ T cells can be induced to kill tumor cell lines and release interferon-γ, tumor necrosis factor-α, interleukin-2 and interleukin-4. Analysis of Vγ and Vδ chain usage indicated that $V\delta3^{+}$ cells are expanded in normal livers (21.2% of γδ T cells) compared to blood (0.5%; $P = 0.001$). Tumor-bearing livers had significant expansions and depletions of γδ T cell subsets but normal cytolytic activity. This study identifies novel populations of liver T cells that may play a role in immunity against tumors.

Keywords: Tumor immunity; Liver; Human; γδ T cells; Cytokines; Cytotoxicity

Introduction

γδ T cells account for small numbers of peripheral blood T cells but accumulate at epithelial surfaces and at sites of infection [1]. Most γδ T cells are negative for CD4 and CD8 and are capable of recognizing antigen without the need for major histocompatibility complex (MHC) restriction (reviewed in Refs. [2,3]. Their T cell receptors (TCRs) recognize relatively conserved structures on pathogens and host cells including non-peptide metabolites and heat shock proteins. Peripheral blood γδ T cells can rapidly lyse antigen-bearing target cells and can kill a range of tumor cell lines in vitro. They also can rapidly release Th1 or Th2 cytokines which can regulate differentiation and activation of components of the adaptive immune system. γδ T cells are therefore thought to bridge innate and adaptive immune responses [2,3].

Distinct subsets of γδ T cells, based on their TCR γ- and δ-chain variable gene segment usage, are differentially distributed in different tissues and show dramatic changes with age. In adult humans, the Vγ9Vδ2 TCR is predominantly found among peripheral blood γδ T cells, whereas the majority of intestinal γδ T cells express Vδ1 chains associated with one of several γ-chains [4–6]. Murine and human studies have provided evidence that the selective accumulations of γδ T cell subsets at different body locations are the result of peripheral selection and expansion by locally expressed antigens [4,7,8]. Other studies, however, have suggested that selection is not required to produce invariant γδ TCRs and that these rearrangements are programmed [9,10]. Vγ9Vδ2 T cells recognize organic phosphoesters, alkylamines, and nucleotide conjugates that are constitutively expressed by host cells and microbial pathogens [11–13]. They also recognize heat shock proteins and putative tumor antigens [14–16]. $V\delta1^{+}$ γδ T cells can recognize the stress-

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inducible MICA and MICB molecules that are upregulated as a result of tumor transformation and virus infection [17,18] although it appears that this recognition is not mediated by the TCR [19]. Vδ1$^+$ T cells have also been shown to recognize the lipid-presenting molecule, CD1c [20].

The liver appears to have an important immunoregulatory role, being able to selectively induce immunity or tolerance to antigens [21]. The adult human liver contains several populations of lymphocytes that exhibit rapid antigen-non-specific cytotoxicity and Th1/Th2 and regulatory cytokine secretion [22–24]. These include many γδ T cells. Here, we have quantified and characterized hepatic γδ T cells from normal and tumor-bearing human liver specimens, with respect to Vγ and Vδ-chain usage, T and NK cell marker expression, cytokine secretion, and cytotoxic potential. Our data indicate that the liver has unique repertoires of γδ T cells that include cells capable of antitumor cytotoxicity in vitro and whose numbers are changed in patients with hepatic malignancy.

Materials and methods

Tissue specimens

Wedge liver biopsies (50–100 mg) were obtained from 11 healthy donor organs (8 male and 3 female; mean age 39.5 years; age range 18–63 years) at the time of liver transplantation. Liver biochemistry and histology were normal in all cases. Liver tissue was obtained from 13 patients undergoing resection for hepatic metastases of colonic origin (7 male and 6 female; mean age 60.2 years; age range 35–75 years). Wedge biopsies from tumor-bearing tissue were taken approximately 10 cm from the tumor margin and appeared histologically normal. All organ donors and patients were negative for hepatitis A, B, C, δ, and E viruses. Ethical approval for this study was obtained from the Research and Ethics Committee at St. Vincent's University Hospital, Dublin, Ireland. Single cell suspensions of hepatic mononuclear cells (HMC) were prepared as previously described [25]. γδ T cells were isolated from fresh HMC preparations using mAb-coated magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's protocols. The purity of isolated fractions was assessed by flow cytometry and only preparations with purities of 95% or greater were used for functional studies.

Antibodies and flow cytometry

Monoclonal antibodies (mAb) specific for human CD3 (clone SK7), CD4 (SK3), CD8αβ (SK1), CD56 (NCAM16.2), γδTCR (11F2), αβTCR (WT31), CD161 (DX12), IFN-γ (25723.11), TNF-α (6401.1111), IL-2 (5344.1111), or IL-4 (8D4-8) were obtained from Becton Dickinson (Oxford, UK). Unconjugated mAbs specific for TCR V-regions Vδ1 (clone 9R.12), Vδ2 (Immu389), Vδ3 (P11.5B), Vγ2-3-4 (23D12), Vγ8 (R4.5), and Vγ9 (Immu360) were obtained from Immunotech (Marseille, France). The expression of these cell surface antigens by freshly isolated HMC and peripheral blood mononuclear cells (PBMC) was detected by mAb staining and three-color flow cytometry (FACScan, Becton Dickinson) and analyzed using CellQuest software (Becton Dickinson). To avoid competition for binding sites on the γδ TCR, the mAb that recognizes all γδ TCRs was not used in conjunction with mAbs specific for individual Vγ or Vδ TCR chains. Instead, cells were costained with the pan-γδ and anti-CD3 mAbs, and anti-CD3 with individual Vγ or Vδ mAbs in separate tubes.

Detection of intracellular cytokines

Cytokine production by γδ T cells after stimulation in vitro was examined by flow cytometry. To eliminate non-specific autofluorescence from other liver cell populations, γδ T cells were first purified from total HMC by immunomagnetic bead separation. Hepatic γδ T cells (1×10^6 cells/ml) were stimulated with 10 ng/ml phorbol myristate acetate (PMA) (Sigma Co., Poole, UK) plus 1 μg/ml ionomycin (Sigma) for 4 h in 24-well plates at 37°C in 5% CO_2. As controls, unstimulated cells were treated similarly. Brefeldin A (10 μg/ml, Sigma), an inhibitor of protein translocation from the endoplasmic reticulum to the Golgi apparatus, was added to the cells for the 4-h incubation. Cells were then stained with mAbs specific for surface CD3 and intracellular IFN-γ, TNF-α, IL-2, or IL-4 and detected by three-color flow cytometry as described previously [24].

Cytotoxicity assays

Magnetic bead-purified γδ T cells were used as effector cells in cytotoxicity assays against K562 and Daudi target cell lines. Natural cytotoxicity and lymphokine-activated killing (LAK) were examined using γδ T cells that were precultured for 3 days in the absence or presence of 50 units/ml IL-2, respectively. Cytotoxicity was assessed in standard 4-h ^{51}Cr release assays using 2000 ^{51}Cr-labeled target cells incubated with purified γδ T cells at E/T ratios of 1:1, 5:1, 25:1, and 50:1 [24]. Specific lysis was calculated from the amounts of ^{51}Cr released into supernatants using the formula: % specific lysis = (cpm of sample − cpm of spontaneous release) × 100 / (cpm of maximum release − cpm of spontaneous release). Spontaneous release was determined by incubating the target cells in the absence of effector cells and maximum release was obtained by incubation of targets with 0.1% Triton X-100.

Statistical analysis

Results were expressed as median and ranges. The Spearman Rank and Mann–Whitney *U* tests for non-parametric data were used to analyze results. *P* values of <0.05 were considered to be significant.

Results

γδ T cells accumulate in human liver

Flow cytometry was used to determine the frequencies of human T cells that express the TCR γδ heterodimer in 11 histologically normal donor livers and matched blood samples (Fig. 1A). For this part of the study, cells were stained ex vivo with no prior manipulation. Fig. 1B shows that there is a significant enrichment of γδ T cells in normal human liver relative to blood, since this TCR was found to be expressed by a median of 0.93% (range 0.24–2.7%) of peripheral blood $CD3^+$ cells and 6.6% (range 4.0–13.8%) of hepatic $CD3^+$ cells ($P = 0.008$).

Phenotypic characterization of γδ T cells in normal human liver

The phenotypes of γδ T cells in freshly isolated matched liver and peripheral blood samples were further analyzed by three-color flow cytometry. Similar to circulating γδ T cells, the majority of hepatic γδ T cells were negative for CD4 and CD8 (median 85.5%; range 79.1–92.0%; Table 1). Less than 5% of hepatic γδ T cells expressed CD4 (4.2%; range 0.8–12.6%), while a significantly higher proportion of hepatic γδ T cells were $CD8^+$ (8.9%; range 6.1%–15.8%) compared with those found in the circulation (1.5%; range 0.6–2.8%) ($P < 0.001$; Table 1). A high proportion of both circulating and hepatic γδ T cells expressed the NK cell markers CD56 (38.9%; range 21.3–57.9% in blood and 38.8%; range 21.1–58.4% in liver) and CD161 (36.8%; range 9.1–59.2% in blood and 36.2%; range 17.0–54.6% in liver; Table 1).

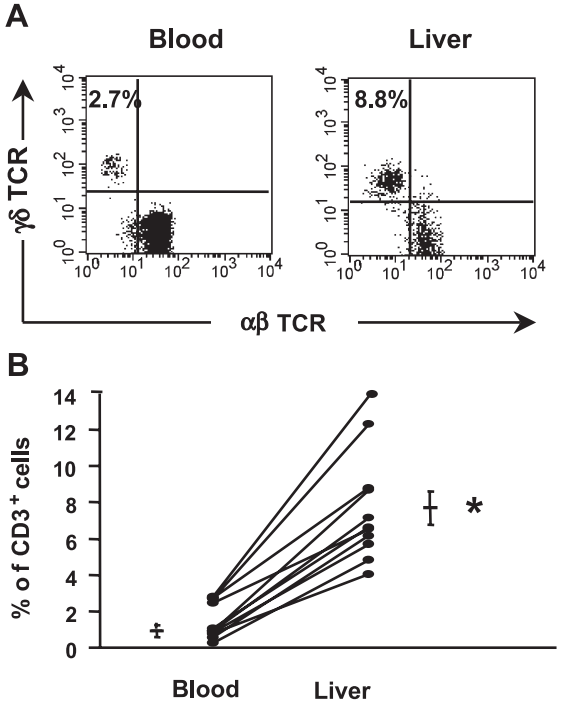

Fig. 1. γδ T cell receptor (TCR) expression by freshly isolated human peripheral blood and hepatic T cells. (A) Representative flow cytometry dot plot showing αβ and γδ TCR expression by gated $CD3^+$ mononuclear cells from the blood and liver of a liver transplant donor. Numbers show the percentages of $CD3^+$ cells that express γδ TCRs. (B) Percentages of $CD3^+$ cells in blood and livers of 11 liver transplant donors expressing γδ TCRs. Medians are shown as horizontal lines with standard errors as error bars. *$P = 0.008$.

Table 1
Phenotypic analysis of peripheral blood and hepatic γδ T cells in 11 histologically normal liver transplant donors

Phenotype	Frequency in blood		Frequency in liver		*P* value
	%	Range	%	Range	
CD4	3.6	0.8–5.4	4.2	0.8–12.6	
CD8	1.5	0.6–2.8	8.9	6.1–15.8	0.001
DN	92.8	91.9–98.7	85.5	79.1–92.0	
CD56	38.8	21.3–57.9	38.9	21.1–58.4	
CD161	36.8	9.1–59.2	36.2	17.0–54.6	

TCR γ and δ chain gene segment usage

To determine whether hepatic γδ T cells display any 'tissue tropism', we examined the expression by hepatic and peripheral blood γδ T cells of the Vγ and Vδ chains that have previously been reported to be the most frequently found in blood, namely Vγ2-3-4, Vγ8, Vγ9, Vδ1, Vδ2, and Vδ3 chains [26,27] (Fig. 2). The Vγ2-3-4, Vγ8, and Vγ9 chains were found to be expressed by similar frequencies of γδ T cells in blood and liver (Fig. 2A). The majority of hepatic γδ T cells expressed Vδ2 chains (56.4%; range 29.0–86.4%) as found in blood, while use of the Vδ1 chain by hepatic γδ T cells (8.9%; range 3.4–32.7%) was significantly reduced compared with that seen in the blood (42.7%; range 5.7–74.2%; $P = 0.035$; Fig. 2A). Strikingly, a significant proportion of hepatic γδ T cells were found to express the Vδ3 chain (21.2%; range 0–37.2%). In contrast, this δ-chain is almost never expressed by circulating lymphocytes (0.5%; range 0–0.9%; $P = 0.001$; Figs. 2A and B).

Cytokine production by hepatic γδ T cells

Flow cytometry was used to examine the cytokine secretion profiles of hepatic γδ T cells in response to in vitro stimulation with PMA and ionomycin (Fig. 3). To enhance clarity of mAb staining, γδ T cells were first positively selected from total HMC using magnetic beads. Hepatic γδ T cells displayed a strong Th1 bias. Significant proportions (median 29.2%; range 14.6–54.9%) of hepatic γδ T cells produced IFN-γ in response to stimulation in vitro with PMA and ionomycin. Smaller proportions produced IL-2 (12.9%; range 8.0–17.1%) or TNF-α (5.3%; range 1.4–15.6%). A median of 4.6% (2.8–6.2%) of hepatic γδ T cells produced the Th2 cytokine IL-4. In addition, 6.2% (1.7–14.7%) of hepatic γδ T cells from healthy liver

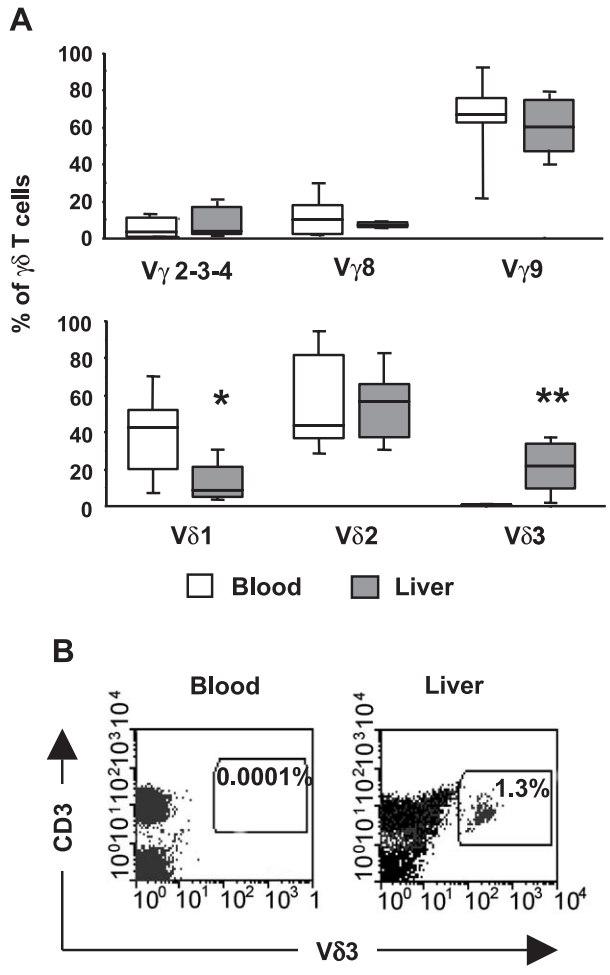

Fig. 2. T cell receptor γ and δ chain usage by freshly isolated human peripheral blood and hepatic T cells. (A) Box plots showing medians (horizontal lines), interquartile ranges (shaded areas) and ranges (error bars) of percentages of γδ T cells in blood and livers of 11 liver transplant donors expressing Vδ1, Vδ2, Vδ3, Vγ2-3-4, Vγ8, and Vγ9. *P = 0.035; **P = 0.001. (B) Representative flow cytometry dot plot showing Vδ3 expression by $CD3^+$ T cells from the blood and liver of a liver transplant donor. The numbers show the percentages of $CD3^+$ T cells that express Vδ3.

spontaneously produced IFN-γ in the absence of stimulation while 7.3% (0.5–12.1%) of unstimulated cells produced IL-2. This spontaneous cytokine production may be due to background levels of in vivo activation, or alternatively, it may be due to low level activation by the mAb-coated magnetic beads used for γδ T cell purification.

γδ T cells in tumor-bearing liver

The numbers, phenotypes, and functions of hepatic and peripheral blood γδ T cells were also compared between liver donors and patients with hepatic malignancy. Fig. 4A shows that γδ T cells are significantly expanded in the blood of patients with hepatic malignancy (3.9% of total $CD3^+$ cells vs. 0.9%; P = 0.008). While tumor-bearing liver also showed increased proportions of γδ T cells (10.4% vs. 6.6%) this increase was not significant (P = 0.3). The expression of the NK cell markers CD56 and CD161 by hepatic γδ T cells was increased in malignancy. CD56 was expressed by a median of 38.9% of γδ T cells from histologically normal livers and 59.7% in malignant tissue (P = 0.04). CD161 was expressed by 36.2% of γδ T cells from normal livers and 47.5% of γδ T cell from tumor-bearing livers (P = 0.05; Fig. 4B).

Analysis of Vγ and Vδ chain usage in tumor-bearing liver revealed significantly different usage compared with healthy liver (Fig. 4C). Tumor-bearing liver displayed preferential usage of the Vδ1 gene products (26.8% of total γδ T cells; P = 0.03), while Vδ2 (21.2%) and Vδ3 (9.61%) expression was significantly reduced (P = 0.03 and 0.02, respectively). While almost all γδ T cells in the blood and liver of liver transplant donors consistently stained positive for mAbs specific for either Vδ1, Vδ2, or Vδ3, approximately 40% of γδ T cells in tumor-bearing livers did not stain for any of these mAbs, suggesting that a γδ T cell with a previously unrecognized TCR δ-chain is expanded in the livers of patients with hepatic malignancy. No differences were seen between Vγ chain usage between normal and tumor-bearing livers (data not shown).

Cytotoxic activity of hepatic γδ T cells

γδ T cells from healthy (n = 4) and tumor-bearing (n = 4) liver samples were purified from freshly isolated HMC using immunomagnetic beads (Fig. 5A) and used as effec-

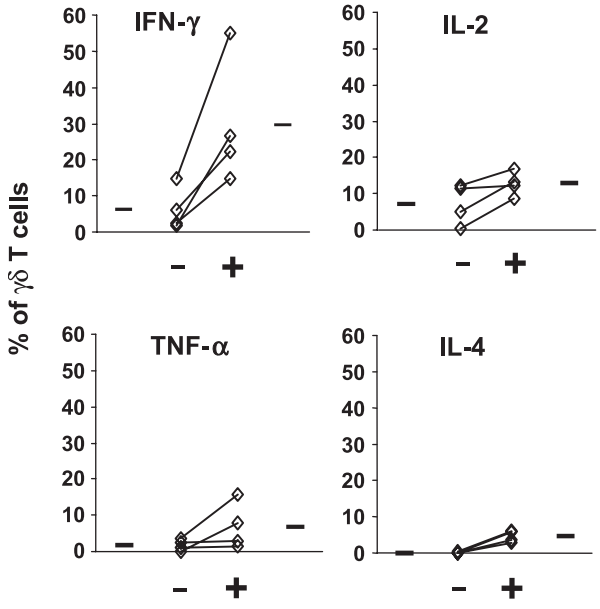

Fig. 3. Cytokine production by hepatic γδ T cells. Percentages of hepatic γδ T cells that stain positive for interferon-γ (IFN-γ), interleukin-2 (IL-2), tumor necrosis factor-α (TNF-α) and IL-4 which were either unstimulated (−) or stimulated for 4 h with PMA and ionomycin (+). Horizontal lines indicate medians of four individual samples.

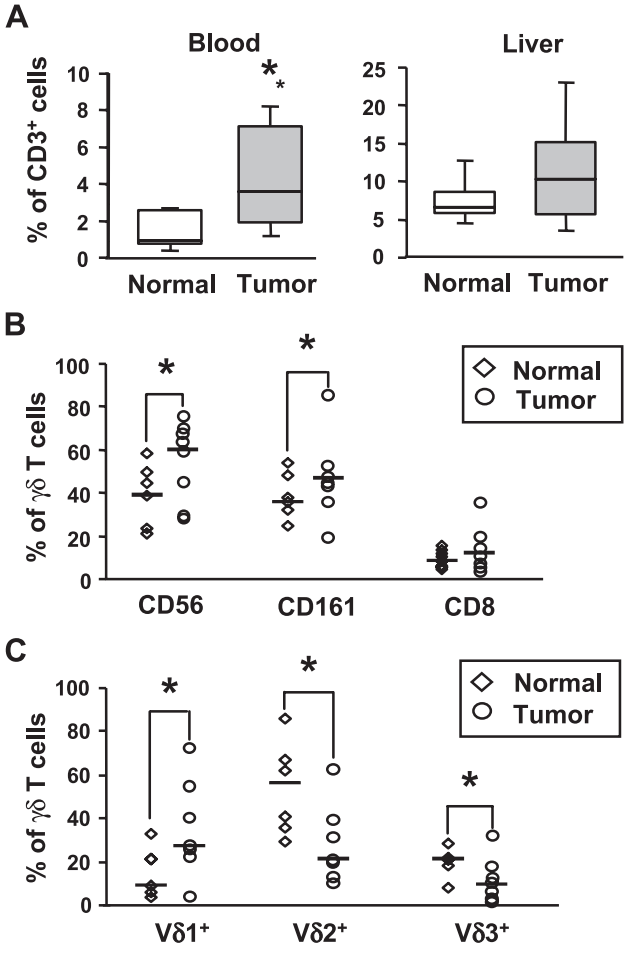

Fig. 4. Peripheral blood and hepatic γδ T cell phenotypes in patients with hepatic malignancy. (A) Box plots showing medians (horizontal lines), interquartile ranges (shaded areas) and ranges (error bars) of percentages of $CD3^+$ T cells in blood and livers of 11 histologically normal and 13 tumor-bearing liver specimens that express γδ T cell receptors. (B and C) Expression of CD56, CD161 and CD8 (B) and Vδ1, Vδ2 and Vδ3 T cell receptor chains (C) by γδ T cells from the livers of 11 histologically normal and 13 tumor-bearing liver specimens. $*P < 0.05$ in all cases.

tors in cytotoxicity assays against K562 and Daudi target cells. When freshly isolated γδ T cells were used as effectors, they were unable to lyse either target cell line (Fig. 5B). However, after incubation for 3 days with 50 units/ml IL-2, γδ T cells from both normal and tumor-bearing livers exhibited cytotoxicity against K562 and Daudi targets (Fig. 5B), indicating that hepatic γδ T cell population contain precursors of LAK cells. No significant difference in the cytotoxic activity of γδ T cells from normal or tumor-bearing livers against K562 or Daudi target cells was observed.

Discussion

γδ T cells are present in significant numbers in the epithelia of the small intestine, skin, lungs and the pregnant uterus but they represent only a small proportion (<5%) of the T cells in peripheral blood [2,3]. The results of the present study indicate that the adult liver also is a site of accumulation of γδ T cells and that this organ has a distinct distribution of the most common TCR γ- and δ-chains. The majority of hepatic γδ T cells express double-negative $CD4^-CD8^-$ phenotypes in keeping with their ability to recognize antigen without the need for MHC-restriction, but $CD4^+$ and $CD8^+$ γδ T cells were also seen. A significant proportion express the NK cell-associated receptors (NKR) CD56 and CD161. Hepatic γδ T cells can be induced by culture with IL-2 to kill the tumor cell lines, K562 and Daudi, and upon activation in vitro, they produce a range of cytokines, including IFN-γ, TNF-α, IL-2, and IL-4.

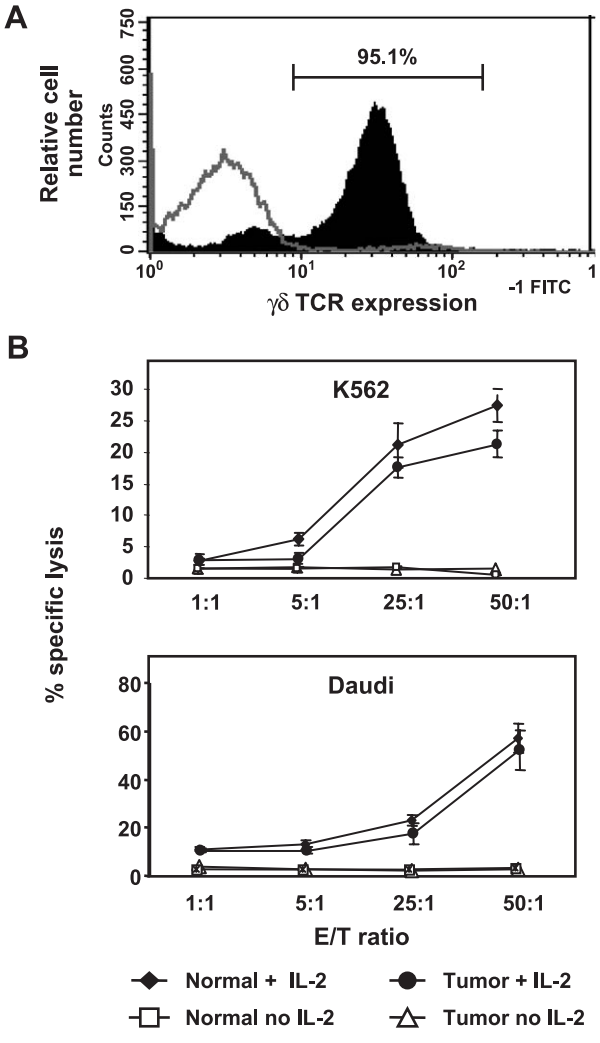

Fig. 5. Hepatic γδ T cells are capable of lymphokine-activated killing of K562 and Daudi target cells. (A) Representative flow cytometry histogram showing γδ T cell receptor (TCR) expression by magnetic bead isolated hepatic γδ T cells (shaded histogram) and unseparated hepatic mononuclear cells (unshaded). The number indicates the purity of the γδ T cell preparation. (B) Natural cytotoxicity (no IL-2) and lymphokine activated killing (+IL-2) of K562 and Daudi target cells by magnetic bead-purified γδ T cells. The figure is representative of experiments using four histologically normal and four tumor-bearing liver samples.

T cells that express NKRs and display potent MHC-unrestricted cytotoxicity and rapid cytokine secretion account for about one third of all hepatic lymphocytes but <5% of peripheral blood lymphocytes [22–24]. These include CD1d-restricted NKT cells with invariant αβ TCRs that recognize lipid antigens [28,29]. Here, we show that they also include γδ T cells. Thus, multiple types of semi-invariant T cells that mediate immediate and regulatory immune functions accumulate in the liver.

In contrast to the human αβ TCR repertoire, the germline encoded γδ TCR repertoire is small [26,30,31]. As previously reported [4,26,27], we found that almost all peripheral blood and hepatic γδ T cells are positive for either Vγ2-3-4, Vγ8, or Vγ9 and either Vδ1, Vδ2, or Vδ3 TCR chains. In both tissues, the majority (usually between 50% and 90%) of γδ T cells express Vγ9 and Vδ2. However, we found that $V\delta3^+$ T cells account for approximately 20% of hepatic γδ T cells but only about 0.5% of peripheral γδ T cells in healthy individuals. The reason for the extensive use of the Vδ3 element in the liver is unclear. It is known that the Vδ3 gene segment frequently recombines with Jα and Cα gene segments and that the Vδ3 TCR chain does not appear to selectively pair with any particular Vγ chain [26]. γδ TCR rearrangements occur early in childhood and particular γδ T cell subsets accumulate at particular body locations with age [4–6]. Such accumulations may be programmed [9,10] or antigen-driven [7,8,27] and if the latter is true, the Vδ3 TCR chain is likely to respond to an antigen found in the liver. $V\delta3^+$ T cells are reported to expand in the peripheral blood of renal transplant recipients that develop cytomegalovirus infection [27]. It is unknown whether $V\delta3^+$ T cells have functions that are different from those of other γδ T cells but previous studies have suggested that different γδ T cells subsets have distinct cytokine secretion patterns [3]. While the biased usage of γδ TCRs in the liver will reflect a distinct specificity of antigen recognition by γδ T cells, future studies using purified $V\delta3^+$ T cells are required to identify their functions.

There is a large body of evidence to suggest that γδ T cells play a role in host defence against tumors [32,33]. γδ T cells display antitumor cytotoxicity in vitro [34–36]. $V\gamma9V\delta2^+$ T cells recognize antigens found on myeloma and lymphoma cells [15,16] and $V\delta1^+$ T cells receive costimulatory signals through NKG2D, a receptor for the nonclassical MHC class I-molecule, MICA, which is upregulated on tumor cells of epithelial origin [17,18]. γδ T cells are present among tumor infiltrating lymphocytes in breast carcinoma [37,38], renal carcinoma [39] pancreatic cancer [40], epithelial tumors [41], melanoma, and sarcoma [42]. Additionally, different patterns of Vδ gene rearrangement have been observed in paired blood and tumor-infiltrating lymphocyte samples from patients with different cancers [43]. Seki et al. [44,45] reported that activated γδ T cells accumulate in the livers of mice and humans with tumors. We have found small overall expansions of γδ T cells expressing NKRs in the blood and livers of patients with hepatic malignancy. However, on examination of γδ T cell subsets, we found that the proportions of hepatic γδ T cells expressing the $V\delta1^+$ TCR chain, thought to play a role in the response to epithelial tumors [17,18], are significantly expanded in the liver. $V\delta1^+$ T cells are also expanded in the livers of patients with chronic hepatitis C virus infection [46], a condition that carries an increased risk of developing hepatocellular carcinoma. In contrast, $V\delta2^+$ cells which are the most abundant hepatic γδ T cell subset, and $V\delta3^+$ cells which appear to be unique to the liver were found in significantly lower frequencies in tumor-bearing livers. Interestingly, the proportions of γδ T cells expressing Vδ1, Vδ2, or Vδ3 TCR chains in the blood and liver of liver transplant donors consistently added up to approximately 100%, but approximately 40% of γδ T cells in tumor-bearing livers did not stain positive for any of these mAbs, suggesting that a γδ T cell with a previously unrecognized TCR δ-chain is expanded in the livers of patients with hepatic malignancy. It is unclear from the present study whether the decreases in $V\delta2^+$ and $V\delta3^+$ cell numbers in tumor-bearing livers are a result of influxes or expansions of $V\delta1^+$ and other γδ T cells. It is possible that changes in the numbers of γδ T cell subsets may either predispose an individual to metastatic liver disease or occur in response to the tumor. Although the proportions of γδ T cell subsets were different in histologically normal and tumor-bearing livers, total γδ T cells purified from these tissues displayed similar LAK cytotoxic activities against K562 and Daudi targets in vitro.

In conclusion, the results of the present study provide further evidence for the presence of cytotoxic and Th1/Th2 cytokine-releasing T cells with semi-invariant TCRs in the adult human liver. Hepatic γδ T cells are predominantly Th1-like cells and show biased TCR usage compared to blood. Our data suggest a role for hepatic γδ T cells in immunity against tumors.

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