

## Distinct subpopulations of $\gamma\delta$ T cells are present in normal and tumor-bearing human liver

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### Abstract

$\gamma\delta$  T cells are thought to mediate immune responses at epithelial surfaces. We have quantified and characterized hepatic and peripheral blood  $\gamma\delta$  T cells from 11 normal and 13 unresolved tumor-bearing human liver specimens.  $\gamma\delta$  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  $\gamma\delta$  T cells can be induced to kill tumor cell lines and release interferon- $\gamma$ , tumor necrosis factor- $\alpha$ , interleukin-2 and interleukin-4. Analysis of V $\gamma$  and V $\delta$  chain usage indicated that V $\delta 3^+$  cells are expanded in normal livers (21.2% of  $\gamma\delta$  T cells) compared to blood (0.5%;  $P = 0.001$ ). Tumor-bearing livers had significant expansions and depletions of  $\gamma\delta$  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.

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### Introduction

$\gamma\delta$  T cells account for small numbers of peripheral blood T cells but accumulate at epithelial surfaces and at sites of infection [1]. Most  $\gamma\delta$  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  $\gamma\delta$  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 compo-

nents of the adaptive immune system.  $\gamma\delta$  T cells are therefore thought to bridge innate and adaptive immune responses [2,3].

Distinct subsets of  $\gamma\delta$  T cells, based on their TCR  $\gamma$ - and  $\delta$ -chain variable gene segment usage, are differentially distributed in different tissues and show dramatic changes with age. In adult humans, the V $\gamma 9$ V $\delta 2$  TCR is predominantly found among peripheral blood  $\gamma\delta$  T cells, whereas the majority of intestinal  $\gamma\delta$  T cells express V $\delta 1$  chains associated with one of several  $\gamma$ -chains [4–6]. Murine and human studies have provided evidence that the selective accumulations of  $\gamma\delta$  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  $\gamma\delta$  TCRs and that these rearrangements are programmed [9,10]. V $\gamma 9$ V $\delta 2$  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 $\delta 1^+$   $\gamma\delta$  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]. V $\delta$ 1<sup>+</sup> 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  $\gamma\delta$  T cells. Here, we have quantified and characterized hepatic  $\gamma\delta$  T cells from normal and tumor-bearing human liver specimens, with respect to V $\gamma$  and V $\delta$ -chain usage, T and NK cell marker expression, cytokine secretion, and cytotoxic potential. Our data indicate that the liver has unique repertoires of  $\gamma\delta$  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,  $\delta$ , 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].  $\gamma\delta$  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), CD8 $\alpha\beta$  (SK1), CD56 (NCAM16.2),  $\gamma\delta$ TCR (11F2),  $\alpha\beta$ TCR (WT31), CD161 (DX12), IFN- $\gamma$  (25723.11), TNF- $\alpha$  (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 V $\delta$ 1 (clone 9R.12), V $\delta$ 2 (Immu389), V $\delta$ 3

(P11.5B), V $\gamma$ 2-3-4 (23D12), V $\gamma$ 8 (R4.5), and V $\gamma$ 9 (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 (FACSscan, Becton Dickinson) and analyzed using CellQuest software (Becton Dickinson). To avoid competition for binding sites on the  $\gamma\delta$  TCR, the mAb that recognizes all  $\gamma\delta$  TCRs was not used in conjunction with mAbs specific for individual V $\gamma$  or V $\delta$  TCR chains. Instead, cells were costained with the pan- $\gamma\delta$  and anti-CD3 mAbs, and anti-CD3 with individual V $\gamma$  or V $\delta$  mAbs in separate tubes.

### *Detection of intracellular cytokines*

Cytokine production by  $\gamma\delta$  T cells after stimulation in vitro was examined by flow cytometry. To eliminate non-specific autofluorescence from other liver cell populations,  $\gamma\delta$  T cells were first purified from total HMC by immunomagnetic bead separation. Hepatic  $\gamma\delta$  T cells ( $1 \times 10^6$  cells/ml) were stimulated with 10 ng/ml phorbol myristate acetate (PMA) (Sigma Co., Poole, UK) plus 1  $\mu$ g/ml ionomycin (Sigma) for 4 h in 24-well plates at 37°C in 5% CO<sub>2</sub>. As controls, unstimulated cells were treated similarly. Brefeldin A (10  $\mu$ g/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- $\gamma$ , TNF- $\alpha$ , IL-2, or IL-4 and detected by three-color flow cytometry as described previously [24].

### *Cytotoxicity assays*

Magnetic bead-purified  $\gamma\delta$  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  $\gamma\delta$  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 <sup>51</sup>Cr release assays using 2000 <sup>51</sup>Cr-labeled target cells incubated with purified  $\gamma\delta$  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 <sup>51</sup>Cr released into supernatants using the formula: % specific lysis = (cpm of sample – cpm of spontaneous release)  $\times$  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

### $\gamma\delta$ T cells accumulate in human liver

Flow cytometry was used to determine the frequencies of human T cells that express the TCR  $\gamma\delta$  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  $\gamma\delta$  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<sup>+</sup> cells and 6.6% (range 4.0–13.8%) of hepatic CD3<sup>+</sup> cells ( $P = 0.008$ ).

### Phenotypic characterization of $\gamma\delta$ T cells in normal human liver

The phenotypes of  $\gamma\delta$  T cells in freshly isolated matched liver and peripheral blood samples were further analyzed by three-color flow cytometry. Similar to circulating  $\gamma\delta$  T cells, the majority of hepatic  $\gamma\delta$  T cells were negative for CD4 and CD8 (median 85.5%; range 79.1–92.0%; Table 1). Less than 5% of hepatic  $\gamma\delta$  T cells expressed CD4 (4.2%; range 0.8–12.6%), while a significantly higher proportion of hepatic  $\gamma\delta$

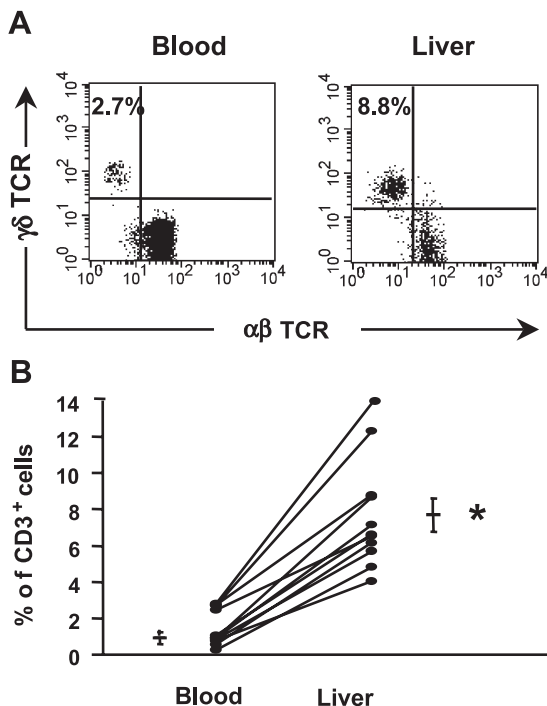


Fig. 1.  $\gamma\delta$  T cell receptor (TCR) expression by freshly isolated human peripheral blood and hepatic T cells. (A) Representative flow cytometry dot plot showing  $\alpha\beta$  and  $\gamma\delta$  TCR expression by gated CD3<sup>+</sup> mononuclear cells from the blood and liver of a liver transplant donor. Numbers show the percentages of CD3<sup>+</sup> cells that express  $\gamma\delta$  TCRs. (B) Percentages of CD3<sup>+</sup> cells in blood and livers of 11 liver transplant donors expressing  $\gamma\delta$  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  $\gamma\delta$  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	0.001
CD8	1.5	0.6–2.8	8.9	6.1–15.8	
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	

T cells were CD8<sup>+</sup> (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  $\gamma\delta$  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).

### TCR $\gamma$ and $\delta$ chain gene segment usage

To determine whether hepatic  $\gamma\delta$  T cells display any 'tissue tropism', we examined the expression by hepatic and peripheral blood  $\gamma\delta$  T cells of the V $\gamma$  and V $\delta$  chains that have previously been reported to be the most frequently found in blood, namely V $\gamma$ 2-3-4, V $\gamma$ 8, V $\gamma$ 9, V $\delta$ 1, V $\delta$ 2, and V $\delta$ 3 chains [26,27] (Fig. 2). The V $\gamma$ 2-3-4, V $\gamma$ 8, and V $\gamma$ 9 chains were found to be expressed by similar frequencies of  $\gamma\delta$  T cells in blood and liver (Fig. 2A). The majority of hepatic  $\gamma\delta$  T cells expressed V $\delta$ 2 chains (56.4%; range 29.0–86.4%) as found in blood, while use of the V $\delta$ 1 chain by hepatic  $\gamma\delta$  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  $\gamma\delta$  T cells were found to express the V $\delta$ 3 chain (21.2%; range 0–37.2%). In contrast, this  $\delta$ -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 $\gamma\delta$ T cells

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

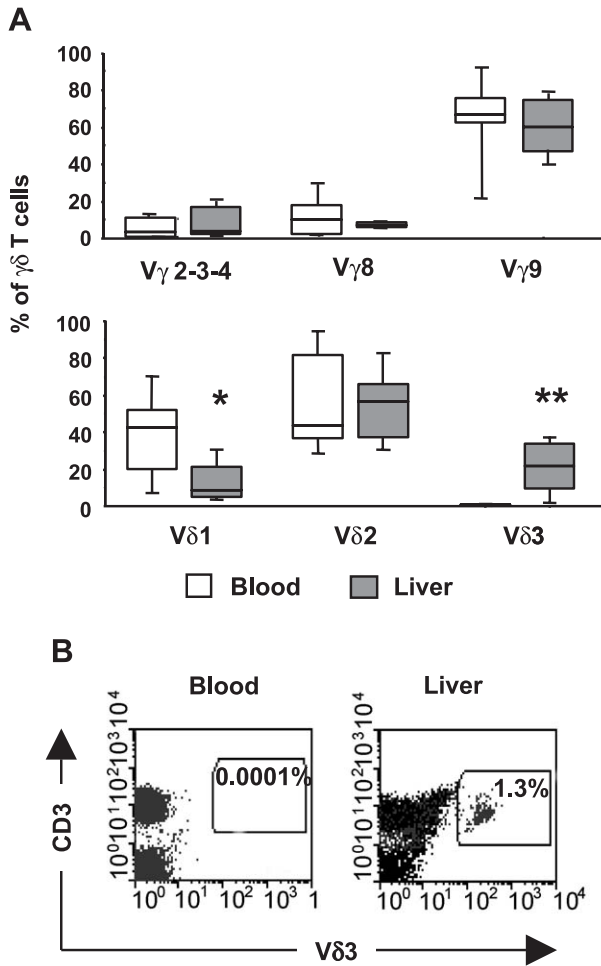


Fig. 2. T cell receptor  $\gamma$  and  $\delta$  chain usage by freshly isolated human peripheral blood and hepatic T cells. (A) Box plots showing medians (horizontal lines), interquartile ranges (shaded areas) and ranges (error bars) of percentages of  $\gamma\delta$  T cells in blood and livers of 11 liver transplant donors expressing V $\delta$ 1, V $\delta$ 2, V $\delta$ 3, V $\gamma$ 2-3-4, V $\gamma$ 8, and V $\gamma$ 9. \* $P = 0.035$ ; \*\* $P = 0.001$ . (B) Representative flow cytometry dot plot showing V $\delta$ 3 expression by CD3<sup>+</sup> T cells from the blood and liver of a liver transplant donor. The numbers show the percentages of CD3<sup>+</sup> T cells that express V $\delta$ 3.

spontaneously produced IFN- $\gamma$  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  $\gamma\delta$  T cell purification.

*$\gamma\delta$  T cells in tumor-bearing liver*

The numbers, phenotypes, and functions of hepatic and peripheral blood  $\gamma\delta$  T cells were also compared between liver donors and patients with hepatic malignancy. Fig. 4A shows that  $\gamma\delta$  T cells are significantly expanded in the blood of patients with hepatic malignancy (3.9% of total CD3<sup>+</sup> cells vs. 0.9%;  $P = 0.008$ ). While tumor-bearing liver also showed increased proportions of  $\gamma\delta$  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  $\gamma\delta$  T cells was increased in malignancy. CD56 was expressed by a median of 38.9% of  $\gamma\delta$  T cells from histologically normal livers and 59.7% in malignant tissue ( $P = 0.04$ ). CD161 was expressed by 36.2% of  $\gamma\delta$  T cells from normal livers and 47.5% of  $\gamma\delta$  T cell from tumor-bearing livers ( $P = 0.05$ ; Fig. 4B).

Analysis of V $\gamma$  and V $\delta$  chain usage in tumor-bearing liver revealed significantly different usage compared with healthy liver (Fig. 4C). Tumor-bearing liver displayed preferential usage of the V $\delta$ 1 gene products (26.8% of total  $\gamma\delta$  T cells;  $P = 0.03$ ), while V $\delta$ 2 (21.2%) and V $\delta$ 3 (9.61%) expression was significantly reduced ( $P = 0.03$  and 0.02, respectively). While almost all  $\gamma\delta$  T cells in the blood and liver of liver transplant donors consistently stained positive for mAbs specific for either V $\delta$ 1, V $\delta$ 2, or V $\delta$ 3, approximately 40% of  $\gamma\delta$  T cells in tumor-bearing livers did not stain for any of these mAbs, suggesting that a  $\gamma\delta$  T cell with a previously unrecognized TCR  $\delta$ -chain is expanded in the livers of patients with hepatic malignancy. No differences were seen between V $\gamma$  chain usage between normal and tumor-bearing livers (data not shown).

*Cytotoxic activity of hepatic  $\gamma\delta$  T cells*

$\gamma\delta$  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-

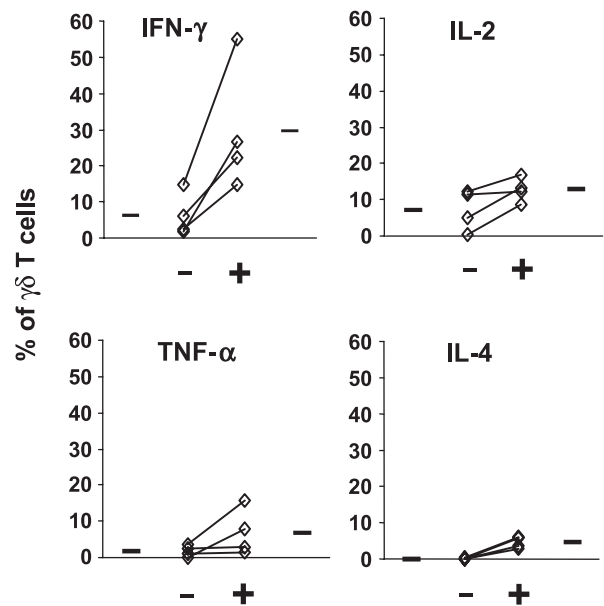


Fig. 3. Cytokine production by hepatic  $\gamma\delta$  T cells. Percentages of hepatic  $\gamma\delta$  T cells that stain positive for interferon- $\gamma$  (IFN- $\gamma$ ), interleukin-2 (IL-2), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and IL-4 which were either unstimulated (–) or stimulated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

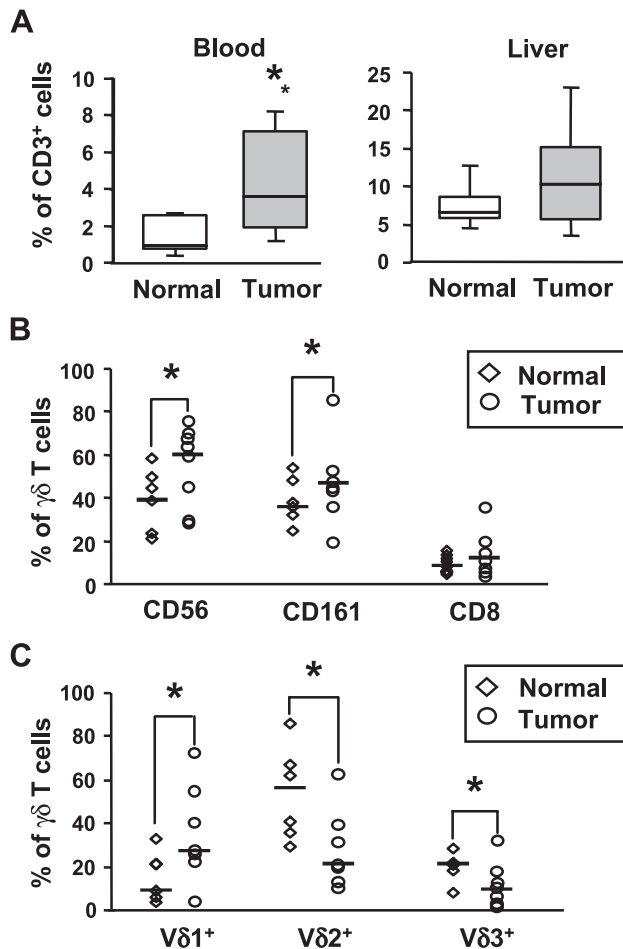


Fig. 4. Peripheral blood and hepatic  $\gamma\delta$  T cell phenotypes in patients with hepatic malignancy. (A) Box plots showing medians (horizontal lines), interquartile ranges (shaded areas) and ranges (error bars) of percentages of CD3<sup>+</sup> T cells in blood and livers of 11 histologically normal and 13 tumor-bearing liver specimens that express  $\gamma\delta$  T cell receptors. (B and C) Expression of CD56, CD161 and CD8 (B) and V $\delta$ 1, V $\delta$ 2 and V $\delta$ 3 T cell receptor chains (C) by  $\gamma\delta$  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  $\gamma\delta$  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,  $\gamma\delta$  T cells from both normal and tumor-bearing livers exhibited cytotoxicity against K562 and Daudi targets (Fig. 5B), indicating that hepatic  $\gamma\delta$  T cell population contain precursors of LAK cells. No significant difference in the cytotoxic activity of  $\gamma\delta$  T cells from normal or tumor-bearing livers against K562 or Daudi target cells was observed.

## Discussion

$\gamma\delta$  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  $\gamma\delta$  T cells and that this organ has a distinct distribution of the most common TCR  $\gamma$ - and  $\delta$ -chains. The majority of hepatic  $\gamma\delta$  T cells express double-negative CD4<sup>-</sup>CD8<sup>-</sup> phenotypes in keeping with their ability to recognize antigen without the need for MHC-restriction, but CD4<sup>+</sup> and CD8<sup>+</sup>  $\gamma\delta$  T cells were also seen. A significant proportion express the NK cell-associated receptors (NKR) CD56 and CD161. Hepatic  $\gamma\delta$  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- $\gamma$ , TNF- $\alpha$ , IL-2, and IL-4.

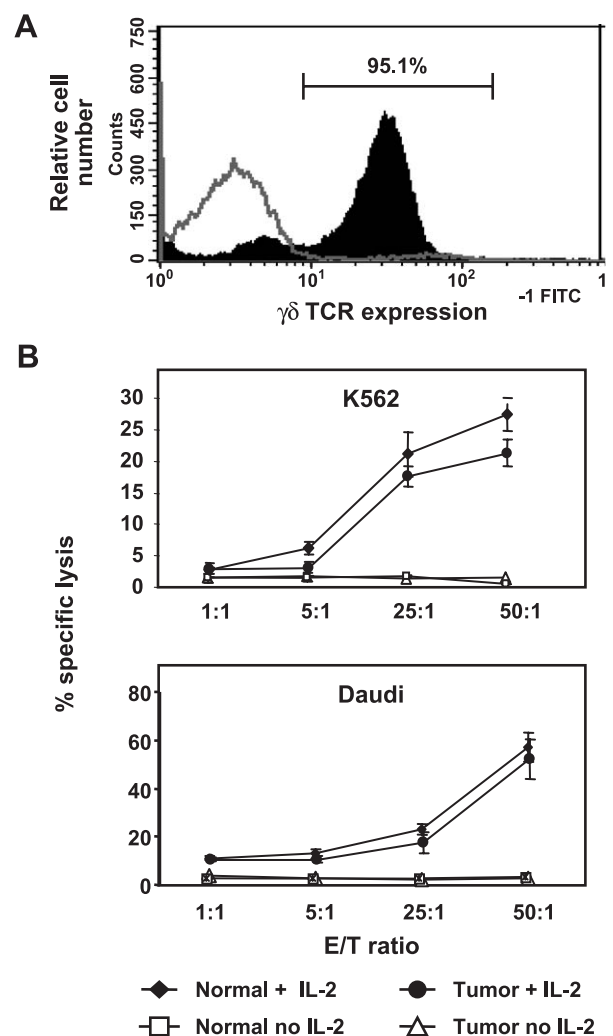


Fig. 5. Hepatic  $\gamma\delta$  T cells are capable of lymphokine-activated killing of K562 and Daudi target cells. (A) Representative flow cytometry histogram showing  $\gamma\delta$  T cell receptor (TCR) expression by magnetic bead isolated hepatic  $\gamma\delta$  T cells (shaded histogram) and unseparated hepatic mononuclear cells (unshaded). The number indicates the purity of the  $\gamma\delta$  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  $\gamma\delta$  T cells. The figure is representative of experiments using four histologically normal and four tumor-bearing liver samples.

T cells that express NKR 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  $\alpha\beta$  TCRs that recognize lipid antigens [28,29]. Here, we show that they also include  $\gamma\delta$  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  $\alpha\beta$  TCR repertoire, the germline encoded  $\gamma\delta$  TCR repertoire is small [26,30,31]. As previously reported [4,26,27], we found that almost all peripheral blood and hepatic  $\gamma\delta$  T cells are positive for either V $\gamma$ 2-3-4, V $\gamma$ 8, or V $\gamma$ 9 and either V $\delta$ 1, V $\delta$ 2, or V $\delta$ 3 TCR chains. In both tissues, the majority (usually between 50% and 90%) of  $\gamma\delta$  T cells express V $\gamma$ 9 and V $\delta$ 2. However, we found that V $\delta$ 3<sup>+</sup> T cells account for approximately 20% of hepatic  $\gamma\delta$  T cells but only about 0.5% of peripheral  $\gamma\delta$  T cells in healthy individuals. The reason for the extensive use of the V $\delta$ 3 element in the liver is unclear. It is known that the V $\delta$ 3 gene segment frequently recombines with J $\alpha$  and C $\alpha$  gene segments and that the V $\delta$ 3 TCR chain does not appear to selectively pair with any particular V $\gamma$  chain [26].  $\gamma\delta$  TCR rearrangements occur early in childhood and particular  $\gamma\delta$  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 V $\delta$ 3 TCR chain is likely to respond to an antigen found in the liver. V $\delta$ 3<sup>+</sup> T cells are reported to expand in the peripheral blood of renal transplant recipients that develop cytomegalovirus infection [27]. It is unknown whether V $\delta$ 3<sup>+</sup> T cells have functions that are different from those of other  $\gamma\delta$  T cells but previous studies have suggested that different  $\gamma\delta$  T cell subsets have distinct cytokine secretion patterns [3]. While the biased usage of  $\gamma\delta$  TCRs in the liver will reflect a distinct specificity of antigen recognition by  $\gamma\delta$  T cells, future studies using purified V $\delta$ 3<sup>+</sup> T cells are required to identify their functions.

There is a large body of evidence to suggest that  $\gamma\delta$  T cells play a role in host defence against tumors [32,33].  $\gamma\delta$  T cells display antitumor cytotoxicity in vitro [34–36]. V $\gamma$ 9V $\delta$ 2<sup>+</sup> T cells recognize antigens found on myeloma and lymphoma cells [15,16] and V $\delta$ 1<sup>+</sup> T cells receive costimulatory signals through NKG2D, a receptor for the nonclassical MHC class I-molecule, MICA, which is up-regulated on tumor cells of epithelial origin [17,18].  $\gamma\delta$  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 V $\delta$  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  $\gamma\delta$  T cells accumulate in the livers of mice and humans with tumors. We have found small overall expansions of  $\gamma\delta$  T cells expressing NKR in the blood and livers of patients with

hepatic malignancy. However, on examination of  $\gamma\delta$  T cell subsets, we found that the proportions of hepatic  $\gamma\delta$  T cells expressing the V $\delta$ 1<sup>+</sup> TCR chain, thought to play a role in the response to epithelial tumors [17,18], are significantly expanded in the liver. V $\delta$ 1<sup>+</sup> 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 $\delta$ 2<sup>+</sup> cells which are the most abundant hepatic  $\gamma\delta$  T cell subset, and V $\delta$ 3<sup>+</sup> cells which appear to be unique to the liver were found in significantly lower frequencies in tumor-bearing livers. Interestingly, the proportions of  $\gamma\delta$  T cells expressing V $\delta$ 1, V $\delta$ 2, or V $\delta$ 3 TCR chains in the blood and liver of liver transplant donors consistently added up to approximately 100%, but approximately 40% of  $\gamma\delta$  T cells in tumor-bearing livers did not stain positive for any of these mAbs, suggesting that a  $\gamma\delta$  T cell with a previously unrecognized TCR  $\delta$ -chain is expanded in the livers of patients with hepatic malignancy. It is unclear from the present study whether the decreases in V $\delta$ 2<sup>+</sup> and V $\delta$ 3<sup>+</sup> cell numbers in tumor-bearing livers are a result of influxes or expansions of V $\delta$ 1<sup>+</sup> and other  $\gamma\delta$  T cells. It is possible that changes in the numbers of  $\gamma\delta$  T cell subsets may either predispose an individual to metastatic liver disease or occur in response to the tumor. Although the proportions of  $\gamma\delta$  T cell subsets were different in histologically normal and tumor-bearing livers, total  $\gamma\delta$  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  $\gamma\delta$  T cells are predominantly Th1-like cells and show biased TCR usage compared to blood. Our data suggest a role for hepatic  $\gamma\delta$  T cells in immunity against tumors.

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