

# Human Intestinal V $\delta$ 1<sup>+</sup> T Lymphocytes Recognize Tumor Cells of Epithelial Origin

By Markus J. Maeurer,<sup>\*‡</sup> Dina Martin,<sup>‡</sup> Wolfgang Walter,<sup>‡</sup>  
Kaihong Liu,<sup>||</sup> Laurence Zitvogel,<sup>‡§</sup> Kathy Haluszczak,<sup>‡</sup>  
Hanna Rabinowich,<sup>§</sup> Rene Duquesnoy,<sup>||</sup> Walter Storkus,<sup>‡§</sup>  
and Michael T. Lotze<sup>‡§</sup>

From the <sup>\*</sup>Department of Medical Microbiology, University of Mainz, D-55101 Mainz, Germany; the <sup>‡</sup>Departments of Surgery, Biochemistry, and Molecular Genetics, the <sup>§</sup>University of Pittsburgh Cancer Institute, and the <sup>||</sup>Division of Transplant Pathology, Department of Surgery, University of Pittsburgh Medical School, Pittsburgh, Pennsylvania 15261

## Summary

$\gamma\delta$  T cells can be grouped into discrete subsets based upon their expression of T cell receptor (TCR) variable (V) region families, their tissue distribution, and their specificity. V $\delta$ 2<sup>+</sup> T cells constitute the majority of  $\gamma\delta$  T cells in peripheral blood whereas V $\delta$ 1<sup>+</sup> T cells reside preferentially in skin epithelium and in the intestine.  $\gamma\delta$  T cells are envisioned as first line host defense mechanisms capable of providing a source of immune effector T cells and immunomodulating cytokines such as interleukin (IL) 4 or interferon (IFN)  $\gamma$ . We describe here the fine specificity of three distinct  $\gamma\delta$ <sup>+</sup> tumor-infiltrating lymphocytes (TIL) obtained from patients with primary or metastatic colorectal cancer, that could be readily expanded in vitro in the presence of IL-1 $\beta$  and IL-7. Irrespective of donor, these individual  $\gamma\delta$  T cells exhibited a similar pattern of reactivity defined by recognition of autologous and allogeneic colorectal cancer cells, renal cell cancer, pancreatic cancer, and a freshly isolated explant from human intestine as measured by cytolytic T cell responses and by IFN- $\gamma$  release. In contrast, tumors of alternate histologies were not lysed, including lung cancer, squamous cell cancer, as well as the natural/lymphocyte-activated killer cell-sensitive hematopoietic cell lines T2, C1R, or Daudi. The cell line K562 was only poorly lysed when compared with colorectal cancer targets. Target cell reactivity mediated by V $\delta$ 1<sup>+</sup> T cells was partially blocked with Abs directed against the TCR, the  $\beta$ 2 or  $\beta$ 7 integrin chains, or fibronectin receptor. Marker analysis using flow cytometry revealed that all three  $\gamma\delta$  T cell lines exhibit a similar phenotype. Analysis of the  $\gamma\delta$  TCR junctional suggested exclusive usage of the V $\delta$ 1/D $\delta$ 3/J $\delta$ 1 TCR segments with extensive ( $\leq$ 29 bp) N/P region diversity. T cell recognition of target cells did not appear to be major histocompatibility complex restricted or to be correlated with target cell expression of heat-shock proteins. Based on the ability of some epithelial tumors, including colorectal, pancreatic, and renal cell cancers to effectively cold target inhibit the lysis of colorectal cancer cell lines by these V $\delta$ 1<sup>+</sup> T cell lines, we suggest that intestinal V $\delta$ 1<sup>+</sup> T cells are capable of recognizing cell surface Ag(s) shared by tumors of epithelial origin.

Two distinct populations of  $\gamma\delta$ <sup>+</sup> TCR-bearing cells have been previously characterized based on their expression of distinct TCR V gene segments and their unique tissue distribution. The dominant  $\gamma\delta$ <sup>+</sup> T cell population in the peripheral circulation is defined by coexpression of the V $\delta$ 2 and the V $\gamma$ 9 TCR (1, 2) segments, constituting 70–90% of the  $\gamma\delta$ <sup>+</sup> T subsets in PBL, and representing 5–10% of all TCR<sup>+</sup> peripheral T lymphocytes. A different, far less frequent,  $\gamma\delta$ <sup>+</sup> T cell population in PBL is detected by the mAb  $\delta$ TCS-1 (3) recognizing the V $\delta$ 1/J $\delta$ 1/J $\delta$ 2 TCR (4). This particular subpopulation constitutes only

5–10% of the  $\gamma\delta$  T cell population in human PBL, but it represents the majority of  $\gamma\delta$  T lymphocytes found in the oral and intestinal epithelia (5–9). The TCR V $\delta$ 2/V $\gamma$ 9 predominance in PBL is thought to reflect an Ag-driven expansion potentially involving the superantigen staphylococcal enterotoxin A (10), isopentyl pyrophosphate, and related prenyl pyrophosphate derivatives, components of mycobacterial Ags (11), or cell-derived proteins expressed upon viral infection (12). Individual  $\gamma\delta$  T cell clones have been previously reported to recognize MHC class I and II, CD1c Ags, nonclassical minor MHC Ags, or heat-shock

proteins (HSPs<sup>1</sup>; 13–20). Extensive analysis of the CDR3 region of the  $\gamma\delta$  TCR (21) suggests that the topology of the interaction of the  $\gamma\delta$  TCR with MHC Ags is clearly different from that observed for  $\alpha\beta^+$  T cells, suggesting that the  $\gamma\delta$  TCR may be able to recognize native (i.e., non-MHC presented) protein Ags (12). This hypothesis implies that Ag recognition by  $\gamma\delta^+$  T cells may more closely resemble soluble Ag recognition by Igs, rather than recognition of short peptide fragments presented by MHC molecules to  $\alpha\beta^+$  TCR cells (12, 19, 20). Whereas some targets have been identified for  $V\delta 2^+$  T cells, the Ags recognized by the  $V\delta 1^+$  TCR subset are unknown. Exclusive expansion of this particular subset has been observed in a variety of different diseases:  $V\delta 1^+$  T cells are selectively expanded in vivo in the peripheral blood of patients with EBV or HIV infections (22–24).  $V\delta 1^+$  T cells are also preferentially expanded in the lungs of patients with pulmonary sarcoidosis and in lesions from patients infected with *Mycobacterium leprae* (25), in synovial fluid from patients with rheumatoid arthritis (26, 27), in cerebrospinal fluid from patients with acute multiple sclerosis (28, 29), in intestinal lesions from patients with coeliac disease (30, 31), and in acute or chronic graft rejections in heart transplant patients (32). Preferential expansion and cytotoxic antitumor responses of the  $V\delta 1^+$  T cell subset have been demonstrated in patients with tumors of epithelial origin, including adenocarcinomas of the lung (33, 34) and, most recently, in renal cell cancer (35). Such  $V\delta 1$  TCR<sup>+</sup> T cells exhibit a characteristic recognition pattern, lysing the autologous and allogeneic cell lines with similar histologies (35).  $V\delta 1^+$  T cells lyse, albeit to a lesser extent, the NK-sensitive cell line K562, but not other NK/LAK-sensitive cell lines, and are apparently not MHC restricted (33–35). We report the in vitro expansion of three  $V\delta 1^+$  T cell lines cultured in the presence of IL-1 $\beta$  and IL-7 from tumor-infiltrating lymphocytes (TIL) derived from patients with primary or metastatic colorectal cancer. These  $\gamma\delta$  T cell lines appear to recognize common Ag(s) shared by colorectal, renal cell, and pancreatic cancers defined by both cytolytic T cell reactivity and by IFN- $\gamma$  release. These data suggest that cytotoxic  $V\delta 1^+$  T cells may be critically involved in immune responses against cancer of epithelial origin in situ.

## Materials and Methods

**Cell Lines.** Target cell lines used in cytotoxicity or cytokine release assays were maintained in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100  $\mu$ g/ml streptomycin (GIBCO BRL, Gaithersburg, MD; complete medium [CM]) unless otherwise indicated. Short-term cultures of freshly harvested tumor single cell suspensions from colorectal cancer patients were also grown in CM. C1R or human EBV-transformed B cell lines (S-EBV) expressing the muc 1 molecules have been described previously (36)

and were grown in CM supplemented with 600  $\mu$ g/ml G418 (GIBCO BRL). The breast cancer cell line BT20 and the cell line U937 (MHC class II negative), its MHC class II-positive subclone I 937, the colorectal cancer cell line DLD-1, the pancreatic cancer cell lines pancreas89 and pancreasQGP9 were kind gifts from Dr. O. Finn (University of Pittsburgh Medical School). The colorectal cancer cell lines LS174 and SW480, the Wilms tumor cell line SKNEP-1, and the explant culture (passage 1) from normal human intestine (CRL7136, HS186.INT) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The colorectal cancer cell lines HCT116 and HT29, the squamous cell cancer cell lines PCI-13 and PCI-50 (head and neck cancer), and target cell lines Daudi, Molt-4, and K562 were kindly provided by Dr. Theresa Whiteside, University of Pittsburgh Cancer Institute. The lung cancer cell lines lung201 and lung89 were obtained from Dr. Steven Dobrowolski (University of Pittsburgh Medical School). The melanoma cell line 526 was kindly provided by Dr. S.A. Rosenberg, (National Cancer Institute). The renal cell cancer cell lines NT1973 and NT1257 and the melanoma cell line Mz18 were kindly provided from Dr. Alex Knuth (Krankenhaus Nordwest, Frankfurt, Germany).

**TIL.** TIL from patients 1881 (colon adenocarcinoma), 3481 (colon adenocarcinoma) and 7179 (colon adenocarcinoma metastatic to liver) were generated as described previously (37) by mincing freshly harvested tumor, by digestion with collagenase (Sigma Chemical Co., St. Louis, MO), and by centrifugation over a Ficoll gradient. The gradient interface was collected, washed twice, resuspended in AIM-V medium (GIBCO BRL), supplemented with 1,000 IU/ml rhIL-1 $\beta$  and 1,000 IU/ml rhIL-7 (kindly provided by Dr. Michael Widmer, Immunex, Seattle, WA) and seeded at  $5 \times 10^5$  cells/ml in 24-well tissue culture plates. TIL have never been restimulated in vitro with tumor. They were split if the cell number exceeded  $1.5 \times 10^6$  cells/ml and fed with IL-1 $\beta$ /IL-7 containing AIM-V medium. TIL were tested at various time points after in vitro propagation for the presence of  $\gamma\delta^+$  T lymphocytes by flow cytometry.  $\gamma\delta^+$  TIL were purified by flow cytometric cell sorting using the anti-pan- $\gamma\delta$  TCR mAb TCR $\delta$ 1 (clone 5A6.E9<sup>3</sup>; T-Cell Sciences, Cambridge, MA; see Table 1) and the FACStar<sup>®</sup> (Becton Dickinson & Co., Mountain View, CA) and were then expanded in IL-1 $\beta$ /IL-7-containing AIM-V medium over a period of 3 mo. Highly purified  $\gamma\delta^+$  TIL were used at various time points during extended culture as effector T cells in cytotoxicity or cytokine release assays, as indicated. The purity of the respective  $\gamma\delta$  T cell lines was determined by flow cytometric analysis before each experiment.  $\gamma\delta^+$ -sorted TIL lines exhibited a stable  $\gamma\delta^+$  TCR phenotype (purity >98%) over the 3-mo period tested.

**Cytolytic Assays.** A standard 4-h chromium release assay was used to assess cytolytic recognition of tumor cells by  $\gamma\delta$  TIL lines 1881, 3481, and 7279 using an E/T ratio of 5:1 unless otherwise indicated. LAK cells were generated using 7-d 6,000 IU IL-2-stimulated PBL obtained from donor leupacks as control effector cells. <sup>51</sup>Cr-labeled target cell suspensions were adjusted to  $0.5 \times 10^5$  cells/ml and 100  $\mu$ l of this cell suspension was added to individual assay wells in triplicate determinations. 100  $\mu$ l of TIL effector cells (E/T ratio, 5:1) were then added to experimental wells and plates incubated for 4 h at 37°C. Spontaneous release wells received 100  $\mu$ l of TIL medium (AIM-V) and maximum release wells received 100  $\mu$ l of Triton X-100 (10% vol/vol in water). For blocking studies, anti-T cell reagents (mAbs against TCR, anti - $\beta$ 2 or - $\beta$ 7 integrin chains, or Abs against the human vitronectin receptor  $\alpha$ v $\beta$ 3 (HVR), or the human fibronectin receptor  $\alpha$ 5 $\beta$ 1 (HFR; see Table 1) were used to pretreat effector

<sup>1</sup>Abbreviations used in this paper: CM, complete medium; HFR, human fibronectin receptor; HVR, human vitronectin receptor; RT, reverse transcriptase; TIL, tumor-infiltrating lymphocyte(s).

cells. Cells were seeded in individual assay wells and incubated for 45 min at 4°C with the respective Ab (10 µg/10<sup>4</sup> cells) and the appropriate isotype controls, followed by two washing steps using AIM-V medium. After careful removal of the supernatant, TIL were resuspended in 100 µl AIM-V medium supplemented with IL-1β and IL-7 and the appropriate target cell suspensions were added as indicated in 100 µl. Target single cell suspensions were treated as indicated in blocking experiments using anti-MHC class I and II, anti-CD1, or anti-HSP Abs (see Table 1). For cold target inhibition assays, 100 µl of the <sup>51</sup>Cr-labeled target cells was added to individual assay wells along with 100 µl of the respective cold target single cell suspension at various dilutions as indicated. T lymphocytes were then added (effector TIL/<sup>51</sup>Cr target cells = 5:1) in a volume of 20 µl per assay well and a standard <sup>51</sup>Cr-release assay was performed twice. 100 µl aliquots was harvested from each well and counted in a gamma counter (Pharmacia LKB). Results are reported as percent specific chromium release calculated as: [(Experimental cpm – spontaneous cpm)/(Maximum cpm – spontaneous cpm)] × 100.

**Cytokine Release Assays.** Stimulator cells were irradiated (3,000 Gy) and adjusted to 10<sup>5</sup> cells/ml in CM. 100 µl of this single cell suspension were added to individual wells and incubated with 100 µl single cell suspension containing individual γδ TIL lines (5 × 10<sup>5</sup> cells/ml) in AIM-V. Control wells included stimulator cells alone or T cells alone. After incubation at 37°C for 16 h, supernatants were harvested and stored at –20°C until assayed for IFNγ (R&D Systems Inc., Minneapolis, MN) by ELISA according to the manufacturer's instructions. Sensitivity for IFN-γ is reported as 5 pg/ml in cell culture supernatants.

**Abs and Flow Cytometry.** The specificity of mAbs or polyclonal antisera used in this study is listed in Table 1. The distributors were T-cell Sciences, Becton Dickinson & Co., Coulter Corp. (Hialeah, FL), Amac, Inc. (Westbrook, ME), Upstate Biotechnology, Inc. (Lake Placid, NY), Telios (now GIBCO BRL): GIBCO BRL, Chemicon (Temecula, CA), Mizzzen, (Victoria, Canada), and Dako (Carpinteria, CA). The mAb w6/32 (anti-MHC class I) and mAb L243 (MHC class II, DR) were obtained from the ATCC and were derived as hybridoma culture supernatants. Briefly, cells were incubated for 30 min at 4°C with directly labeled FITC- or PE- conjugated Ab. For some unlabeled Abs, a secondary reagent FITC or PE F(ab')<sub>2</sub> anti-mouse or anti-rabbit IgG was used. Controls included normal mouse or rabbit IgG obtained from Becton Dickinson & Co. prelabeled with FITC or PE. In each flow cytometric sample, 5,000 events were measured and gates were set to exclude nonviable cells. Individual samples were analyzed using a FACScan® (Becton Dickinson & Co.) flow cytometer using the software program Lysis II, and results are reported as percent positive-staining cells evaluated in two-color flow cytometric analysis. Using single-color (FITC or PE)-based detection systems, results are reported as percent positive-staining cells and are depicted as fluorescence intensity versus cell size (FSC) histograms in order to allow for the identification of potential T cell subsets demonstrating differentially dim or bright Ab staining.

**Template cDNA Preparation.** Total RNA from 5 × 10<sup>6</sup> to 5 × 10<sup>7</sup> cells was extracted from γδ TIL lines, from freshly harvested biopsies obtained from colon cancer lesions, or from tumor-free mucosa (adjacent to tumor) obtained from patients undergoing surgery for resection of colorectal cancer at the University of Pittsburgh Medical Center or at the Veterans Administration Hospital (Pittsburgh, PA) using RNazol (Biotecx, Inc., Houston, TX) according to the method of Chomczynski and Sacchi (38). First strand cDNA synthesis was performed by heating the reac-

tion at 37°C for 1 h, followed by 5 min at 95°C using a PCR thermal cycler (Perkin-Elmer Corp., Norwalk, CT). The 40-µl reaction volume contained 8 µg of RNA in 16 µl H<sub>2</sub>O, 8 µl 5× reaction buffer (GIBCO BRL), 4 µl dithiothreitol (final concentration, 10 mM), 2 µl dNTP (dATP, dCTP, dGTP, dTTP, final concentration, 1 mM each), 3 µl RNase inhibitor (final concentration, 120 U), 1 µl actinomycin D (final concentration, 2 µg), 4 µl Oligo-dT random primers (final concentration, 0.8 µg), and 2 µl Moloney murine leukemia virus reverse transcriptase (RT) (Perkin-Elmer Corp., final concentration, 400 IU/ml).

**Amplification of TCR-specific Templates.** 150 ng of cDNA was used in each individual PCR reaction throughout all experiments and the integrity of individual cDNA samples was analyzed using human β-actin-specific primers (5'–3'; forward: ATTTGCGGTGGACGATGGAGGGGC, backward: GGCATCGTCACCAACTGGGACGAC) using the temperature profile 95°C for denaturation, annealing for 1 min at 60°C, followed by 1 min at 72°C for extension (30 cycles). The protocol for amplification of Vδ- or Vγ-specific mRNA transcripts was 95°C (2 min for denaturation) followed by 33 cycles of 94°C (1 min), 59°C (1 min, 30 s), and 72°C (1 min). The primers sequences for specific Vγ or Vδ transcripts have been previously reported elsewhere (28). Generally, each reaction mixture (50 µl vol) contained 25 µl H<sub>2</sub>O, 5 µl PCR 10X reaction buffer (Perkin-Elmer Co.), 8 µl dNTP (concentration, 1.25 mM each), 5 µl of each primer (final concentration, 50 pmol each primer), 1.5 µl cDNA (150 ng), and 1 µl Taq-polymerase (Perkin-Elmer Corp.). The respective PCR reactions were run on 1.5% ethidium bromide-stained agarose gels. In each PCR run, a positive control (cDNA from heat-killed *Mycobacterium tuberculosis*-stimulated PBL) and a negative control (PCR mastermix without template cDNA) were included. Primers were synthesized at the DNA Synthesis Facility, University of Pittsburgh. The identity of individual PCR products was confirmed by Southern blot using internal Vγ- or Vδ-specific primers (data not shown).

**Sequencing of Vδ1+ PCR Products.** Vδ1+ PCR products generated from in vitro-cultured (>98% pure) γδ+ T cell lines were purified from agarose gels using microfilters obtained from Millipore Corp. (Bedford, MA) and subcloned into the TA cloning vector obtained from Invitrogen (San Diego, CA). Positive clones were randomly selected from each individual γδ TIL line and sequenced using the ABI system (Applied Biosystems, Foster City, CA) at the University of Pittsburgh DNA sequencing facility.

## Results

**Colorectal Cancer-derived γδ T Cells Expanded in IL-1β and IL-7 Exclusively Express the Vδ1 TCR Segment and Preferentially Coexpress the Vγ2 TCR.** Tumor-infiltrating T lymphocytes were obtained from three patients with colorectal cancer (patients 1881: primary colon adenocarcinoma; 3481: primary colon adenocarcinoma; 7279: colon adenocarcinoma metastatic to the liver) and were cultured for 6 wk in IL-1β and IL-7 (1,000 IU/ml each) containing AIM-V medium. Phenotypic analysis of individual (bulk) cultures performed at day 54 revealed 10–48% CD3<sup>+</sup>/γδ<sup>+</sup>/Vδ1<sup>+</sup> positive-staining T cells (data not shown). γδ+ T cells were purified from TIL cultures by flow cytometric cell sorting at day 54 using a mAb recognizing a framework epitope present on all γδ TCR. Positively sorted γδ T cells were expanded in CM containing IL-1β and IL-7 for at least

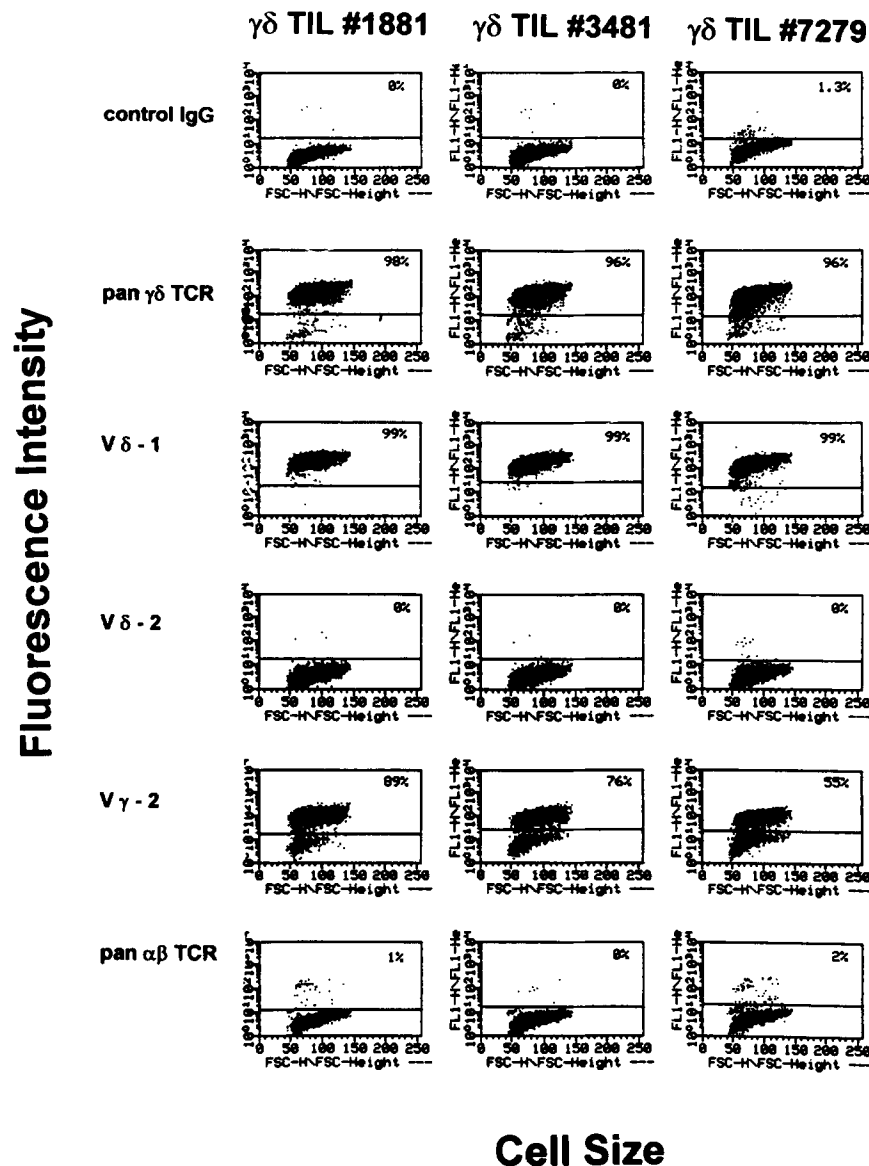
**Table 1.** List of Antibodies Employed for Flow Cytometry or Blocking Experiments

Antibody designation	Specificity	Distributor
WT-31	Framework (pan) $\alpha\beta$ TCR	Becton Dickinson
SA6.E9 <sup>3</sup>	Framework (pan) $\gamma\delta$ TCR	T-cell Diagnostics
$\delta$ TCS-1	V $\delta$ 1/J $\delta$ 1/J $\delta$ 2 TCR segments	T-cell Diagnostics
15D	V $\delta$ 2 TCR segment	T-cell Diagnostics
7A5	V $\gamma$ 2 (V- $\gamma$ 9) TCR segment	T-cell Diagnostics
5T-022	CD1a	CD workshop Ab
5T-024	CD1c	CD workshop Ab
S5.2	LFA-3, CD58 ligand	Becton Dickinson
SK3	CD4	Becton Dickinson
SK1	CD8	Becton Dickinson
G25.2*	CD11a ( $\alpha^L/\beta$ 2), LFA-1	Becton Dickinson
D12*	CD11b ( $\alpha^M/\beta$ 2). C3bi receptor	Becton Dickinson
SHCL-3*	CD11c ( $\alpha^X/\beta$ 2) C3bi,dg receptor	Becton Dickinson
BL5	CD18, $\beta$ 2 chain of integrins	Amac
2A3	CD25, IL-2 (TAC) receptor	Becton Dickinson
K20	CD29, $\beta$ 1 chain of integrins	Amac
Ber-H2	CD30, Ki-I Ag	Dako
L-178	CD44, HCAM, homing receptor	Becton Dickinson
2H4	CD45-RA	Coulter
UCHL-1	CD45-RO	Becton Dickinson
1.2B6	CD62E, ELAM-1, E-selectin	Immunotech (Marseille, France)
Dreg 56	CD62L, MEL14-Ag, L-selectin	Immunotech
L78	CD69, gp34/28	Becton Dickinson
LB-2	CD54, ICAM-1	Chemicon
133	CD80, B7.1 (CD28 ligand)	CD workshop Ab
1331, Fun-1	CD86, B7.2 (CD28 ligand)	Pharmingen
3B1.18,KP43	CD94, $\alpha\beta$ , $\gamma\delta$ , and NK subset	CD workshop Ab
MA-ICAM2-F	CD102, ICAM-2	Endogen, Inc. (Boston, MA)
P1	VLA-1, $\alpha$ 1/ $\beta$ 1, laminin <sup>+</sup> collagen receptor	Telios
P1E6	VLA-2, $\alpha$ 2/ $\beta$ 1, laminin <sup>+</sup> collagen receptor	Telios
P1E5	VLA-3, $\alpha$ 3/ $\beta$ 1, collagen, fibronectin receptor	Telios
B-5G10	VLA-4, $\alpha$ 4/ $\beta$ 1, VCAM-1, fibronectin receptor	Upstate Biotechnology
P1-D6	VLA-5, $\alpha$ 5/ $\beta$ 1, fibronectin receptor	Telios
GoH3	VLA-6, $\alpha$ 5/ $\beta$ 1, laminin receptor	Amac
LM142	$\alpha$ v (CD51), vitronectin receptor chain	Chemicon
LM609	$\alpha$ v $\beta$ 3 (CD51/CD61) vitronectin receptor	Chemicon
P1F6	$\alpha$ v $\beta$ 5, vitronectin receptor	Chemicon
Polyclonal antiserum, absorbed with $\alpha$ 5 $\beta$ 1	$\alpha$ v $\beta$ 3/ $\beta$ 5, vitronectin receptor	GIBCO BRL
Polyclonal antiserum, absorbed with $\alpha$ v $\beta$ 3/ $\beta$ 5	$\alpha$ 5 $\beta$ 1, fibronectin receptor	GIBCO BRL
HML-1	CD103, $\beta$ 7 integrin chain on IEL	Amac and Immunotech
SPA804	HSP 60	Mizzen
SPA826-2, polyclonal antiserum	HSP 68	Mizzen
SPA810	HSP 70	Mizzen
SPA825	HSP 75	Mizzen
SPA811	HSP 75	Mizzen
SPA820	HSP 73	Mizzen

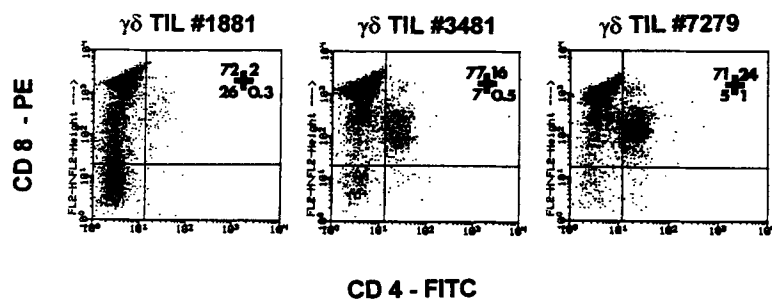
three additional months and those cells displayed a stable phenotype, which was documented on a weekly basis by flow cytometric analysis. Fig. 1 shows a characteristic flow cytometric analysis of sorted  $\gamma\delta^+$  TIL after 2 mo of in vitro expansion. 96–98% of the T cells stained positive with an Ab recognizing all  $\gamma\delta^+$  T cells, and 99% of T cells from three different TIL lines stained positive with a mAb defining the V $\delta$ 1/J $\delta$ 1/J $\delta$ 2<sup>+</sup> subset (mAb  $\delta$ TCS1). TIL stained negative for reactivity with a mAb detecting the V $\delta$ 2<sup>+</sup> T cell subset, which is the most frequent  $\gamma\delta$  T cell population in PBL. The majority of the V $\delta$ 1/J $\delta$ 1<sup>+</sup> TIL population also coordinately stained positive with an Ab recognizing the V $\gamma$ 2<sup>+</sup> subset (or according to an alternate nomenclature, V $\gamma$ 9) staining 89% of cells in TIL 1881, 76% of cells in TIL 3481, and 55% of cells in TIL 7279. The  $\alpha\beta^+$  T cell population represented a minor contaminant of 0.1–2% in individual TIL cultures. To obtain additional characterization

of specific V $\gamma$  usage in such V $\delta$ 1<sup>+</sup> TIL, we evaluated V $\gamma$  usage by RT-PCR using a primer panel specific for V $\delta$ / $\gamma$  subset families. All three  $\gamma\delta$  T cell lines exhibited V $\delta$ 1-specific transcripts by RT-PCR and common usage of the V $\gamma$ 1.2,  $\gamma$ 1.8, and  $\gamma$ 2 chains of the TCR (data not shown).

*In Vitro-cultured  $\gamma\delta^+$  T Lymphocytes Obtained from Different Patients Express a Similar Pattern of Expression of Adhesion Molecules.* Positively selected  $\gamma\delta$  T cells were analyzed for expression of activation markers or adhesion molecules by flow cytometry using a panel of mAbs listed in Table 1. Characteristically, examination of cell surface expression of CD8 and CD4 in these TIL lines indicated the presence of more than one discrete  $\gamma\delta^+$  T cell population showing high and intermediate level of CD8 expression, as well as a population reproducibly characterized by intermediate level of CD8 concomitant with CD4 expression (Fig. 2). These  $\gamma\delta^+$  TIL lines expressed a reproducibly similar pat-



**Figure 1.** Colorectal cancer-derived  $\gamma\delta$  TIL exclusively use the V $\delta$ 1 chain and preferentially coexpress the V $\gamma$ 2 chain of the TCR.  $\gamma\delta^+$  T cells (obtained from patients 1881, 3481, and 7279) were isolated by cell sorting using the anti-pan- $\gamma\delta$  mAb5A6.E9<sup>3</sup>, and cultured for 2 wk in AIM-V medium containing IL1 $\beta$ /IL-7 (1,000 IU/ml) before phenotypic analysis by flow cytometry using mAbs recognizing  $\alpha\beta$  or  $\gamma\delta$  T cell subsets. Positively sorted TIL stained for 98% of the cell population positive with an anti-pan- $\gamma\delta$  TCR mAb or using the V $\delta$ 1/J $\delta$ 1/J $\delta$ 2-specific mAb  $\delta$ TCS-1. The majority of cells expressed the V $\gamma$ 2 chain and stained negative for expression of the V $\delta$ 2 chain or the  $\alpha\beta$  TCR.



**Figure 2.** CD4 and CD8 expression in colorectal cancer  $\gamma\delta^+$  TIL.  $\gamma\delta$  positively-sorted TIL (see Fig. 1) were stained for CD4 and CD8 expression by flow cytometry. The majority of  $\gamma\delta^+$  TIL exhibited the  $CD8^+CD4^-$  phenotype.  $CD4^+CD8^+$  (double positive)  $\gamma\delta$  TIL were repetitively observed in  $\gamma\delta$  TIL lines 3481 and 7279.

tern of reactivity using mAb specific for activation or adhesion molecules (summarized in Table 2), exhibiting a  $CD11a-c^+$ ,  $CD30^+$ ,  $CD44^+$ ,  $CD45RA$  high,  $CD45RO$  low, MHC class II low, IL-2 receptor (TAC) low,  $CD69^+$ , very late Ag (VLA)  $1-6^+$ , vitronectin receptor $^-$ , and fibronectin receptor $^+$  phenotype.

*Colorectal Cancer-derived  $V\delta 1^+$  TIL Exhibit a Similar Pattern of Cellular Reactivity against Colorectal, Pancreatic, and Renal Cell Cancers Defined by Cytolysis and IFN- $\gamma$  Release.*  $\gamma\delta^+$  TIL lines were evaluated at three different times during in vitro culture for cytolytic reactivity against a panel of target cancer cell lines of diverse histological origin (compiled in Fig. 3). TIL release of IFN- $\gamma$  in response to each target was also evaluated for the three  $\gamma\delta$  TIL lines. 7-d LAK cells (PBL cultured in 1,000 IU/ml IL-2) served as control effector cells and efficiently lysed each target cell line ( $>45\%$  lysis at an E/T of 30:1) with the exception of the colorectal cancer cell line HT29 (showing 10% specific lysis).  $\gamma\delta$  TIL lines lysed the NK target cell line K562 poorly (in the range of 8–14% specific lysis) and failed to lyse a variety of the LAK/NK-sensitive target cell lines including Daudi, C1R, U937, Molt-4, and T2. EBV-transformed B cell targets, as well as mucin-expressing EBV $^+$  B cell lines (C1R-Muc1, S-EBV-Muc1) were not lysed by  $\gamma\delta$  TIL. Examination of IFN- $\gamma$  release by individual  $\gamma\delta$  TIL lines in response to these target cells revealed no significant increase in IFN- $\gamma$  secretion compared with the constitutive release of IFN- $\gamma$  in the range of 37–40 pg/ml/16 h (Fig. 3).

Of interest,  $\gamma\delta$  TIL lines 1881, 3481, and 7279 lysed the colorectal cancer cell line 3481 (obtained from patient 3481) in addition to four different allogeneic colorectal cancer cell lines, with the exception of HT29. Examination of TIL release of IFN- $\gamma$  in response to autologous (i.e., in the case of the matched  $\gamma\delta$  TIL line 3481 and tumor line 3481) and allogeneic colorectal cancer cell lines revealed that all three  $\gamma\delta$  TIL lines secreted IFN- $\gamma$  in response to autologous or allogeneic tumor cell lines ( $\leq 273$  pg/ml/16 h), even in the absence of a significant cytolytic response (i.e., in the case of the target cell line HT29). These  $\gamma\delta$  TIL lines did not lyse or secrete significant levels of IFN- $\gamma$ , in response to diverse target cell lines, i.e., melanoma, one breast cancer, lung cancer, or autologous (obtained from patient 3481) or allogeneic fibroblasts. In contrast,  $\gamma\delta$  TIL lines 1881, 3481, and 7279 efficiently lysed two pancreatic

cancer cell lines (pancreas89 and pancreasQGP-1), two renal cell cancer cell lines (RC1257 and RCC1973), the normal human intestine explant culture line (passage 1, CRL7136; ATCC), and to a lesser extent, the Wilms tumor cell line SKNEP-1. However, these  $\gamma\delta$  TIL did not lyse two additional squamous cell cancer cell lines (PCI-13 and PCI-50, data not shown). Target-specific release of IFN- $\gamma$  release by  $V\delta 1^+$  T cells in response to these targets was not significant. Of note, despite some minimal cytotoxic  $\gamma\delta$  TIL responses against K562, we could not detect significantly elevated IFN- $\gamma$  secretion by  $\gamma\delta$  TIL stimulated by K562 (in the range of 40 pg/ml/16 h compared with  $\leq 260$  pg/ml IFN- $\gamma$  release in response to colorectal cancer stimulator cells).  $\gamma\delta$  TIL did not proliferate specifically in response to in vitro stimulation with colorectal cancer cells or tumors of alternate histologies (data not shown).

*The  $\gamma\delta$  TCR, the  $\beta 2$  and  $\beta 7$  Integrin Chains and the Fibronectin Receptor Are Involved in  $\gamma\delta^+$  T Cell-mediated Cytolysis of Tumor Targets.* To evaluate the functional impact of cell surface molecules on cytolytic  $\gamma\delta$  T cell (colorectal cancer cell line 3481) recognition of target cells, we incubated each  $\gamma\delta$  TIL line with Abs directed against the (pan- $\gamma\delta$ ) TCR,  $V\delta 1^+$  TCR,  $V\delta 2^+$  TCR, the HFR, the HVR, or the  $\beta 2$  or  $\beta 7$  integrin chains before addition of  $^{51}Cr$ -labeled target cells in 4-h cytotoxicity assays. The cytolytic T cell recognition of colorectal cancer cell line 3481 was inhibited by  $\sim 30\%$  by the anti-pan- $\gamma\delta$  TCR or the  $V\delta 1/J\delta 1/J\delta 2$ -specific mAbs (Fig. 4). TIL 1881 and 3481 (cytotoxic) recognition of tumor 3481 was similarly inhibited by 35%. Addition of mAb directed against  $V\delta 2$  did not affect T cell-mediated lysis. All three  $\gamma\delta$  TIL lines showed a diminished cytotoxic T cell response if preincubated with antiserum specific for the fibronectin receptor ( $\leq 59\%$  inhibition of specific lysis) but not with antiserum specific for the vitronectin receptor.  $\gamma\delta$  T cell-mediated lysis was inhibited by 80% by an Ab directed against the  $\beta 2$  integrin chain, and by  $\leq 50\%$  by a mAb directed against the  $\beta 7$  integrin chain, a component of the intraepithelial lymphocyte (IEL) marker. This was, however, not a uniform trend since the  $\gamma\delta$  TIL line 3481 was only inhibited by  $\sim 10\%$  by an Ab directed against CD103 (i.e.,  $\beta 7$  chain).

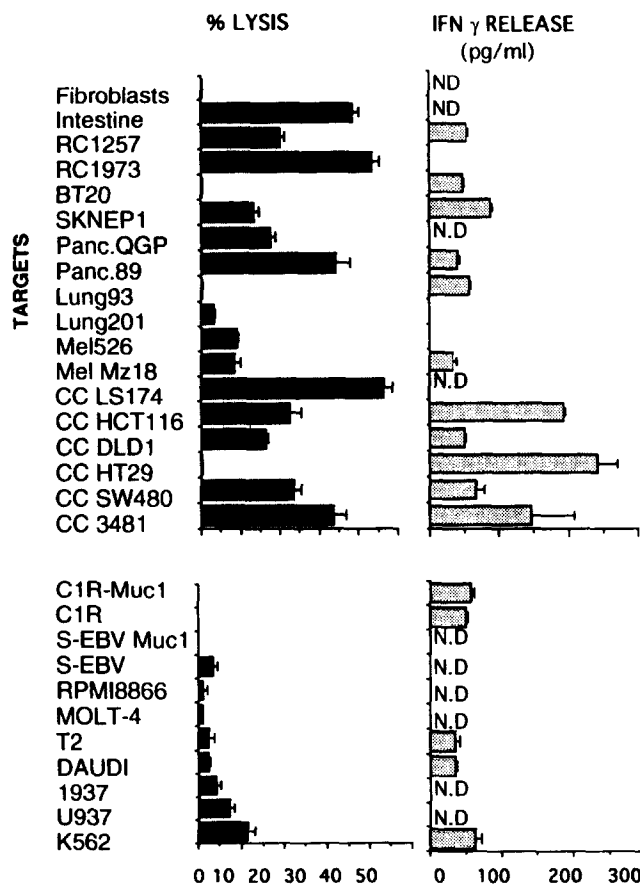
*Colorectal Cancer-derived  $V\delta 1^+$  T Lymphocytes Recognize Cell Surface Ags Shared by Colorectal, Pancreatic, and Renal Cell Cancer Cell Lines.*  $\gamma\delta^+$  TIL lines 1881, 3481, and 7279 appear to recognize both autologous and allogeneic col-

**Table 2.** Phenotypic Analysis of  $\gamma\delta^+$  TIL: Activation Markers and Adhesion Molecules

Percent positive-staining cells for:	$\gamma\delta$ TIL 1881	$\gamma\delta$ TIL 3481	$\gamma\delta$ TIL 7279
		%	
CD2	98	97	98
CD3	99	99	99
CD11a	99	99	99
CD11b	97	97	98
CD11c	84	80	74
CD25 (TAC)	2	2	2
CD30	92	82	77
CD44	90	86	96
CD45RA	91	94	69
CD45RO	14	43	36
HLA-DR	15	6	6
CD69	95	96	95
CD94	0	23	7
ICAM-1	95	90	95
ICAM-2	99	95	96
CD80	4	8	4
CD86	24	34	36
VLA-1	96	95	97
VLA-2	95	93	93
VLA-3	31	19	14
VLA-4	70	98	98
VLA-5	99	93	93
VLA-6	43	60	65
E-Selectin	0	0	0
L-Selectin	0	0	0
$\beta 1$ -integrin chain	99	99	99
$\beta 2$ -integrin chain	99	99	99
$\beta 3$ -integrin chain	2	2	1
$\beta 7$ -integrin chain	95	92	99
$\alpha v$	10	8	2
$\alpha v\beta 3$	2	2	1
$\alpha v\beta 3/\beta 5$ (HVR)	1	1	1
$\alpha v\beta 5$	2	2	1
$\alpha 5\beta 1$ (HFR)	98	95	99

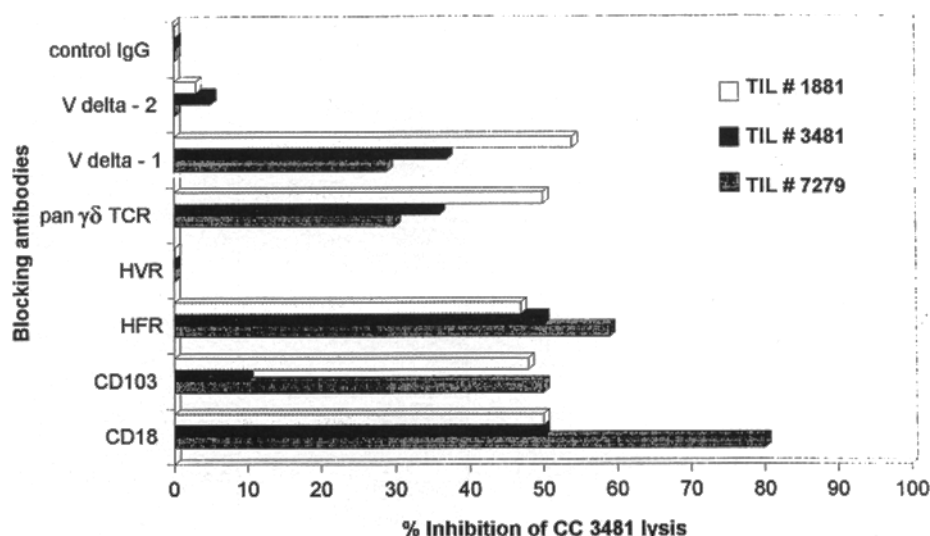
Positive sorted  $\gamma\delta^+$  T cells, analyzed by flow cytometry for expression of activation markers, exhibited a similar phenotype in three different experiments at various time points. Viable lymphocytes were gated based on size (FSC) and granularity (SSC) criteria, and data were collected for 5,000 cells as determined by forward light scatter intensity using Lysis II software on a FACScan<sup>®</sup>. Results are reported as percent of cells staining positive for a given marker.

orectal cancer cell lines, pancreatic cancer, and renal cell cancer (Fig. 3). However, these  $\gamma\delta$  T cell effector cells also recognize (to comparably lesser extent) the erythroleukemia cell line K562 and early passage cells derived from "normal



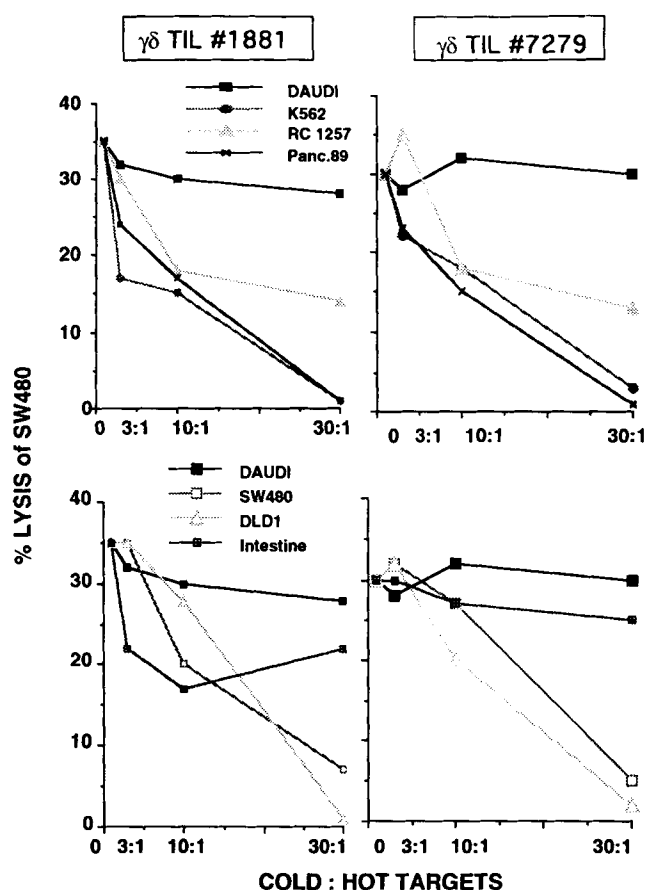
**Figure 3.** Similar target cell recognition pattern of  $V\delta 1^+$  TIL as measured by cytotoxicity and IFN- $\gamma$  release. Colorectal cancer target cell lines and cell lines of alternate histology were tested for  $\gamma\delta$  T cell recognition using a standard 4-h  $^{51}\text{Cr}$ -release assay or using a 16-h IFN- $\gamma$  release assay as described in Materials and Methods. Results obtained from three individual  $\gamma\delta$  TIL lines (1881, 3481, and 7279) are expressed as the mean  $\pm$  SD of percent specific lysis or IFN- $\gamma$  release in response to individual targets. These  $\gamma\delta$  TIL lines preferentially lyse colorectal cancer (CC) cell lines, the human intestinal cell explant CRL7186, pancreatic (Panc.), and renal cell cancer (RC) cell lines. Tumors of alternate origin were not lysed significantly, including two head and neck squamous cell cancer cell lines, allogeneic fibroblasts (data not shown), or autologous fibroblasts (obtained from patient 3481). IFN- $\gamma$  release by  $\gamma\delta$  TIL is reported as pg/ml/ $10^6$  cells/16 h. E/T = 5:1.

intestine" (explant culture CRL7136, HS186.INT; ATCC). We performed cold target inhibition assays using the  $^{51}\text{Cr}$ -labeled allogeneic colorectal cancer cell line SW480 and a panel of nonlabeled target cell lines as competitive inhibitors in order to evaluate the commonality of ligands expressed on these target cells. Cold targets were admixed with  $^{51}\text{Cr}$ -labeled SW480 targets at 30:1, 10:1, and 3:1 (cold/hot target ratios) as indicated (Fig. 5) with two  $\gamma\delta$  TIL lines (1881 and 7279) evaluated as effector cells. Both  $\gamma\delta$  TIL lines efficiently lysed the target cell line SW480 in the absence of cold target inhibitors. Recognition of SW480 targets by TIL effector cells was effectively inhibited by cold targets including the colorectal cancer cell lines SW480 and DLD-1, and pancreatic cancer (pancreas89), renal cell cancer (RC1257),



**Figure 4.**  $\gamma\delta$  TIL cell recognition can be blocked by Abs directed against the  $\beta 2$  or  $\beta 7$  integrin chains, or with Abs directed against the fibronectin receptor.  $\gamma\delta$  TIL lines were incubated at  $4^{\circ}\text{C}$  for 30 min with Abs against the TCR (anti-pan  $\gamma\delta$ ,  $\delta\text{TCS1-V}\delta 1$  and  $V\delta 2$ ), anti- $\beta 2$  (CD18), or  $\beta 7$  (CD103) integrin chains, or with Abs directed against the human vitronectin  $\alpha v\beta 3$  (HVR) or fibronectin  $\alpha 5\beta 1$  (HFR) receptor, or in medium alone. After washing these  $\gamma\delta^{+}$  effector T cells in AIM-V,  $^{51}\text{Cr}$ -labeled colorectal cancer cells (CC 3481, autologous to  $\gamma\delta$  TIL 3481) were added to effector cells and a standard 4-h  $^{51}\text{Cr}$ -release assay was performed. Evaluation of T cell reactivity without blocking Abs (i.e., incubation in medium alone) revealed that  $\gamma\delta$  TIL line 1881 lysed the CC 3481 line to a level of 35%, 3481  $\gamma\delta$  TIL to a level of 30%, and  $\gamma\delta$  7279 exhibited 43% specific lysis of CC 3481 at

an E/T ratio of 10:1. Results are reported as percent inhibition of specific lysis of control CC 3481 (no Ab) by  $\gamma\delta$  TIL. Preincubation of  $\gamma\delta$  TIL with relevant anti-TCR Abs reduced CC 3481-specific recognition in the range of 30–35%. Killing of CC 3481 by  $\gamma\delta$  TIL could also be inhibited using Abs directed against the  $\beta 2$  and the  $\beta 7$  integrin chains, and with Abs directed against the fibronectin receptor (HFR). In contrast, Abs against the vitronectin receptor (HVR), or nonrelevant control IgG, did not efficiently block  $\gamma\delta$  T cell-mediated lysis. Incubation of target cells with these mAbs or with mAbs directed against MHC class I or II molecules did not result in significant inhibition of target cell cytolysis (data not shown).



**Figure 5.**  $V\delta 1^{+}$  TIL lines recognize common target antigens expressed by tumor cells of epithelial origin.  $^{51}\text{Cr}$  labeled SW480 cells (HOT TARGETS) were seeded in 96-well plates and (non- $^{51}\text{Cr}$ -labeled, COLD

and the erythroleukemia K562 cell lines. In contrast, cytolytic  $\gamma\delta$  T cell recognition of  $^{51}\text{Cr}$ -labeled SW480 cells was only marginally inhibited by cold targets such as the Burkitt lymphoma cell line Daudi, or cells derived from normal intestine (CRL7136, HS186.INT; ATCC).

**TCR  $V\delta 1$  Transcripts in  $\gamma\delta$  TIL Lines 1881, 3481, and 7279.** To assess potential preferential usage of  $V\delta 1$  transcripts by individual  $\gamma\delta$  T cell lines (which apparently recognize shared tumor Ags), we extracted mRNA from each TIL line, reverse transcribed it into cDNA, and used  $V\delta 1$ -specific PCR primers (spanning the  $V\delta 1$  segment to the constant region of the TCR) to amplify  $V\delta 1$ -specific products; we subcloned the PCR product and sequenced 8–10 individual clones from each TIL line (Fig. 6). Each subcloned PCR product showed the TCR  $V\delta 1$  region paired exclusively with  $J\delta 1$ . 72% (20/28) of TCR transcripts were in frame and showed high diversity in the CDR3 region. TCR usage of the D $\delta 3$  segment (20/20) compared with

TARGETS) single cell suspension of the cell lines K562, Daudi, the renal cell cancer cell line RC1257, the pancreatic cancer cell line panc89, the colorectal cancer cell lines SW480 and DLD-1, or the human intestinal cell explant culture CRL7186 were added concomitantly at various concentrations resulting in 3:1, 10:1, and 30:1 cold/hot target cell ratios.  $\gamma\delta$  TIL lines 1881 or 7279 were then added to individual assay wells. The effector/(hot) target ratio was 10:1 and a 4-h  $^{51}\text{Cr}$ -release assay was performed.  $\gamma\delta$  TIL 1881 exhibited 35%, and  $\gamma\delta$  TIL 7279 30% specific lysis of CC 3481 target cells in the absence of cold targets.  $\gamma\delta$  TIL recognition of (hot) SW480 cells could be inhibited by cold CC SW480, CC DLD1 targets, by the renal cell cancer line RC1257, the pancreatic cancer cell line panc89, and by K562. In contrast, unlabeled Daudi cell targets did not interfere significantly with CC SW480 target cell recognition by  $\gamma\delta$  TIL lines.



CLONE germ line	IN FRAME	V delta 1	N/P	D1 gaataagt	N/P	D2 cttctctac	N/P	D3 actgggggatacg	N/P	J delta 1 accgcgataaactc
1881-1	+	ctcttgggggaact	aaaaactta	aaat		ctctac	gg	ggggg	ctcgt	cgcataaactc
1881-3	+	ctcttggggga	tgtacgtggatgag					ctgggggatac	a	gataaactc
1881-5	+	ctcttggggga	cacagg			cttctctac	agataaa	ggggg	tgggcgatactgtaaggggcgctcaggt	cgcataaactc
1881-7	+	ctcttgggggaact	agacaagccccggcggggttt					actgg	tac	taaaactc
1881-8	+	ctcttgggggaact	agacggggtaagg			tac	gtca	ggggg	caat	ataaactc
1881-10	+	ctcttg	tcc	aaata	ttcttca	ctttcc	agdtctaccggcgagccccacgaacct	ggggg	gggaaccac	cgcataaactc
1881-11	+	ctcttgggg					g	ctgggggatacg	tt	acacgcgataaactc
3481-2	+	ctcttggg	attcttctctcag					ctgggg	agct	acacgcgataaactc
3481-4	+	ctcttgggg	gctattccc			ctttc	tacgtg	ctgggggata	tggttggt	acacgcgataaactc
3481-7	+	ctcttggggga	taaccagccttccatgtgg					actgggggga	ccaacgggtgg	acacgcgataaactc
3481-AB	+	ctcttggggga	tattcgatccc					tgggg	ccaagatc	actc
3481-AE	+	ctcttgggggaact	ct			ttctctac	gttc	ggg	tgc	cgcataaactc
7279-2	+	ctcttggggga	ct					tggggg	agagggg	aaactc
7279-3	+	ctcttgggggaac	cgtagagag			cta	gg	tgggggat	cttaac	acacgcgataaactc
7279-4	+	ctcttggg	aaacccc			ctttctctac	ctttac	tgggg	tt	acacgcgataaactc
7279-5	+	ctcttgggtgaac	gccagggcagcagagattga			ctttctctac	aac	gggggat	acgcag	acacgcgataaactc
7279-10	+	ctcttgggggaac	acagg					tggggg	ctcaagt	acacgcgataaactc
7279-11	+	ctcttggggg	ggttccggt					actgggggata	gctgggtgc	taaaactc
7279-AA	+	ctcttggg	ccgacagacattacg					tggggg	gcgcgttt	ataaactc
7279-AC	+	ctcttgggggaac	ccccgaacccc					gggggata	accctgggtgt	cgcataaactc

**Figure 6.** Junctional TCR sequences from IL-1 $\beta$ /IL-7-expanded antitumor-reactive  $\gamma\delta^+$  TIL. V $\delta$ 1-specific transcripts were generated by RT-PCR as described in Materials and Methods using primers specific for the V $\delta$ 1 family and the constant region of the  $\delta$  TCR followed by subcloning of individual V $\delta$ 1 transcripts. Numbers indicate the  $\gamma\delta$  TIL lines ( $\gamma\delta$  TIL 1881, 3481, and 7279) and the respective designation of individual cDNA clones. Note the preferential use of J $\delta$ 1 and D $\delta$ 3 with V $\delta$ 1 and extensive rearrangement of the TCR junctional regions. These sequence data are available from Genbank/EMBL/DDBJ under accession numbers U30189-U30208.

the D $\delta$ 2 segment (9/20); 2/20 cDNA clones used the D $\delta$ 1 segment. Nucleotide sequences assigned to the D $\delta$ 1 or the D $\delta$ 2 regions were typically shorter, compared with segments used by D $\delta$ 3. N/P region nucleotides were remarkably long ( $\leq 29$  bp in cDNA clone 1881-10). Fig. 7 depicts the predicted amino acid sequences encoded by each individual cDNA clone. We noted that in 6/20 TCR sequences (3' to the D $\delta$ 3 segment, NP region), a hydrophobic aromatic tyrosine (Y), and in (different) 8/20 TCR sequences, a hydrophilic, aliphatic leucine (L) residue were encoded. The amino acid residue was in some cDNA clones generated by basepairs provided by the V region and the N/P region, and in other cDNA clones from basepairs in the N/P region alone, 3' to the V $\delta$ 1 chain of the TCR.

**TCR Repertoire in Colorectal Cancer.** The V $\delta$ 1<sup>+</sup> T cell population has been previously shown to represent the predominant T cell subset observed in normal human intestine (5–8). Since we did not have normal mucosa or PBL available from patients 1881, 3481, and 7279, we could not directly address whether the mRNA transcripts detected in  $\gamma\delta$  TIL were also present in normal colonic mucosa adjacent to colon cancer or in PBL from these patients. The three  $\gamma\delta$  TIL lines showed a preferential usage of the V $\gamma$ 2, V $\gamma$ 1.2, and V $\gamma$ 1.8 TCR segments (as determined by RT-PCR), which were also exclusively identified in the freshly collected tumor samples obtained from these patients, thereby excluding potential in vitro artefacts associated with preferential T cell subset expansion (data not shown). To test for TCR V $\delta$ /V $\gamma$  usage in matched (primary) colon ad-

enocarcinoma samples and in tumor-free (distal or proximal to the tumor) adjacent mucosa, we extracted RNA from freshly collected tumor/normal mucosa samples obtained from seven additional patients with primary colorectal cancer. mRNA was reverse transcribed into cDNA and tested for TCR  $\gamma$  or  $\delta$  transcripts by RT-PCR as described in Materials and Methods. Specific PCR products were validated by Southern blot analysis using internal primers for individual TCR  $\gamma$  or  $\delta$  transcripts (data not shown). 5/7 adenocarcinoma samples exclusively expressed V $\delta$ 1 transcripts, whereas 2/7 adenocarcinoma samples showed additional V $\delta$ 2 transcripts, which were also found in 7/7 tumor proximal/distal normal mucosa samples (Table 3). 2/7 adenocarcinoma samples exclusively expressed the TCR  $\gamma$ 1.2 and  $\gamma$ 2 transcripts, and 2/7 cancer samples expressed additionally V $\gamma$ 1.8 transcripts (identical to the TCR  $\gamma$  usage pattern in  $\gamma\delta$  TIL lines described above). 3/7 cancer specimens expressed the same V $\gamma$  mRNA expression pattern and additionally expressed V $\gamma$ 3 mRNA. All seven normal (matched) mucosa specimens showed typical V $\delta$ 1 and V $\delta$ 2 mRNA transcripts and predominantly V $\gamma$ 1.2,  $\gamma$ 1.8,  $\gamma$ 2,  $\gamma$ 3, and, in two specimens, V $\gamma$ 1.4 mRNA, expression. In contrast, the matched adjacent tumor tissue revealed exclusive expression of V $\delta$ 1 and V $\gamma$ 1.2, V $\gamma$ 1.8, and V $\gamma$ 2 mRNA (Table 3). Restricted V $\delta$ 1/V $\gamma$ 1.2, V $\gamma$ 1.8, V $\gamma$ 2 mRNA expression patterns were also obtained upon examination of 25 primary colorectal cancer specimens and 15 colon adenocarcinomas metastatic to liver (our unpublished observations).

CLONE	V delta 1	N/P; D1; N/P; D2; N/P; D3; N/P	J delta 1
1881-1	L G	K N L K S Y G G A P S	D K L
1881-3	L G E	L Y V D E L G D T	D K L
1881-5	L G E	H R A F L Q I K G V G D T V K G R Q V	D K L
1881-7	L G E	L D K P P A G F T G T	K L
1881-8	L G E	L D G V R Y V R G Q Y	K L
1881-10	L	V Q I S S P S K S H R R S P R T L G E G T T	D K L
1881-11	L G	G W G I R Y	T D K L
3481-2	L G	I L F S R W G A Y	T D K L
3481-4	L G	D L L R A G G Y G G Y	T D K L
3481-7	L G E	L F H W G T N R W	T D K L
3481-AB	L G	D N P A F H V V G P R S	L
3481-AE	L G E	L F R S R V P	D K L
7279-2	L G	D S L R S G G R G	K L
7279-3	L G E	P W R A R W G I L T	T D K L
7279-4	L G	N P P F L P L L G F	T D K L
7279-5	L G E	R Q A T R L R G Y A D	T D K L
7279-10	L G E	H R P S Y N W G L K Y	T D K L
7279-11	L G	G F R Y W G I A G A	K L
7279-AA	L G	P T D L R G G P L Y	K L
7279-AC	L G E	P P E P R G I T P G V	D K L

**Figure 7.** Predicted junctional amino acid sequences encoded by V $\delta$ 1 transcripts from antitumor-reactive  $\gamma\delta$ <sup>+</sup>TIL. Individual cDNA clone designations correspond to those in Fig. 6. Amino acid sequences are reported using the single letter code. Note that 2/20 cDNA clones exhibit the amino acid L in the N/P region 3' to the V $\delta$ 1 region and 6/20 cDNA clones exhibit the amino acid Y generated by the N/P region 3' to the D $\delta$ 3 segment. Sequence data are available from Genbank/EMBL/DBJ under accession numbers U30189–U30208.

## Discussion

We have successfully expanded three individual colorectal cancer-derived  $\gamma\delta^+$  T cell lines by in vitro culture using IL-1 $\beta$  and IL-7. This strategy was based on previous observations (39) that a combination of IL-1 $\beta$  and IL-7 promotes the preferential outgrowth of peritoneal  $\gamma\delta$  T cells from *Listeria monocytogenes*-infected mice. IL-1 $\beta$  may act directly on  $\gamma\delta^+$  TCR-responsive T cells (39, 40) or IL-1 $\beta$ -mediated effects may be attributed to initial IL-1 $\beta$ -induced activation of dendritic cells, which have recently been shown to be involved in  $\gamma\delta$  T cell activation and expansion (41). The IL-1 $\beta$ /IL-7-expanded TIL populations exhibited stable phenotype and functional characteristics for 4 mo in culture. This phenotypic stability may reflect an inherent characteristic of the  $\gamma\delta$  T cell subset examined, or may be due to IL-7-mediated effects that have been reported to sustain Ag-specific (CD4 $^+$   $\alpha\beta$  T cells) in the absence of antigenic restimulation for  $\leq 22$  mo (42). Cytokine-mediated effects on cell surface expression of adhesion molecules has been well documented for human NK cells that exhibit differential expression of CD11a-c, and VLA1-6 cell upon IL-2 stimulation (43). Alternatively, the similar pattern of adhesion molecule expression in  $\gamma\delta$  T cell lines derived from three individual patients may reflect a tissue-specific (i.e., intestinal) phenomenon. For example,  $\gamma\delta$  T cells isolated from intestinal lymph nodes and Peyer's patches appear to express varied levels of CD44 or B7 molecules that impact their tissue-biased homing pattern (44). Synovial fluid V $\delta 1^+$  T cells from patients with juvenile

rheumatoid arthritis express predominantly the early activation marker CD69 and the high molecular isoform of the CD45 (CD45RA) (45), a pattern that we similarly observed for IL-7-expanded  $\gamma\delta$  T cells in this report.

The bulk  $\gamma\delta$  TIL population obtained from three individual patients showed exclusive expression of the V $\delta 1$  TCR and preferential coexpression of the V $\gamma 2$  TCR. Additional TCR  $\gamma$  transcripts, including those for the V $\gamma 1.2$  and the V $\gamma 1.8$  chains, were detected by RT-PCR. Of note, a similar restricted pattern of such V $\gamma$ /V $\delta$  TCR transcripts was identified in the majority of colon adenocarcinomas as compared with the adjacent tumor-free mucosa (showing additional V $\delta 2$ , and V $\gamma 3$ , and V $\gamma 1.4$  transcripts), indicating that this particular  $\gamma\delta$  T cell population may be preferentially enriched at the tumor site.

$\gamma\delta^+$  T cells appear to represent effector cells capable of specifically recognizing cancer cells (33–35, 46). All previous reports of tumor-reactive  $\gamma\delta^+$  T lymphocytes support the exclusive expression of the V $\delta 1^+$  TCR. V $\delta 1^+$  T cells specifically recognize pancreatic cancer in a MHC-unrestricted fashion and proliferate in response to pancreatic cancer cells in vitro in sufficient numbers that have prompted some authors to suggested the clinical implementation of this T cell subset in the adoptive immunotherapy of pancreatic cancer. Additional reports have demonstrated (non-MHC-restricted and  $\gamma\delta$  TCR-mediated, tumor-reactive) cytotoxic  $\gamma\delta$  T cell responses in TIL derived from lung cancer patients. This T cell subset also exclusively used the V $\delta 1^+$

**Table 3.** V $\gamma$ /V $\delta$  TCR mRNA Transcripts Differ in Colorectal Cancer Lesions Compared with Adjacent Tumor-free Mucosa and Exhibit a Preferential Usage of V $\gamma$  1.2, 1.8, 2, and V $\delta 1^+$  TCR

Sample no.	Histological diagnosis	TCR V $\gamma$ regions						TCR V $\delta$ regions				
		1.2	1.4	1.8	2	3	4	1	2	3	4	5
22	Normal mucosa	1.2	1.4	1.8	2	3		1	2			
21	Adenocarcinoma colon	1.2		1.8	2			1				
84	Normal mucosa	1.2		1.8	2	3		1	2			
82	Adenocarcinoma colon			1.8	2			1				
207	Normal mucosa		1.4	1.8	2	3		1	2			
206	Adenocarcinoma colon			1.8	2			1				
213	Normal mucosa	1.2		1.8	2	3		1	2			
212	Adenocarcinoma colon	1.2		1.8	2	3		1	2			
256	Normal mucosa	1.2		1.8	2	3		1	2			
255	Adenocarcinoma colon	1.2		1.8	2			1				
271	Normal mucosa	1.2		1.8	2	3		1				
270	Adenocarcinoma colon	1.2		1.8	2			1				
285	Normal mucosa	1.2		1.8	2	3		1	2			
284	Adenocarcinoma colon			1.8	2			1				

mRNA was extracted from freshly resected (primary) colorectal cancer specimens and from tumor-free adjacent mucosa harvested 3 cm proximal or distal to the tumor lesions. RNA was reverse transcribed into cDNA and tested for V $\gamma$ /V $\delta$  TCR-specific mRNA transcripts in individual samples by RT-PCR using a primer panel as described in Materials and Methods. Identity of PCR products was confirmed by using V $\gamma$ - or V $\delta$ -specific V-region TCR internal oligonucleotides by Southern blot analysis (data not shown).

TCR (33, 34), a finding that has been corroborated for  $\gamma\delta$  T cell clones obtained from a patient with renal cell cancer (35). In this latter study, the majority of  $V\delta 1^+$  T cell clones preferentially recognized the autologous renal cell cancer, but additional  $V\delta 1^+$  T cell clones from this patient also lysed allogeneic renal cell cancer lines (35).  $V\delta 1^+$  T cells have also been identified in a variety of human diseases that are thought to have an "autoimmune" etiology (26–31), and have also been identified in increased numbers in Wilms tumors (47) and in the liver of tumor-bearing hosts (48). The  $V\delta 1^+$  TIL lines generated in our study lyse colorectal, renal cell, and pancreatic cancer cell lines, an early passage line of human normal intestine (CRL7136, HS186.INT; ATCC), and to a lesser extent the erythroleukemia cell line K562. Our results confirm previous observations that  $V\delta 1^+/V\gamma 2$  (or  $\gamma 9$ ) $^+$  T cells typically do not lyse Daudi targets. Whereas such ( $V\delta 1^+V\gamma 2^+$ ) T cells may recognize an Ag expressed on Burkitt's lymphoma cell lines (49), this specificity does not appear to be relevant in our study. Based on cold target inhibition assays, our  $\gamma\delta$  TIL lines appear to exhibit similar specificities and to recognize common target structures expressed by epithelial target cell lines and freshly explanted/cultured intestinal tissue (Fig. 3). However,  $\gamma\delta$  TIL-mediated lysis of  $^{51}\text{Cr}$ -labeled colorectal cancer cells could not be efficiently inhibited by excess unlabeled normal intestinal cells (Fig. 5) suggesting that different, distinct target structures expressed by colon cancer and intestinal epithelium may be recognized by  $\gamma\delta$  T cells. The observation that a single cell explant culture from normal human intestine is also recognized by such  $\gamma\delta$  T cells may alternatively suggest that the potential target structures for  $\gamma\delta$  T cell recognition may represent normal "self" (i.e., not associated with malignant transformation) Ags with differential TIL reactivity due to differential Ag expression levels on tumors versus normal mucosa. In any case, it is not yet clear, based on the available data, whether potential autoreactive  $\gamma\delta^+$  TIL are functional in vivo (50, 51), or whether such effects are due to the in vitro expansion of effector immune cells in the presence of IL-1 $\beta$  and IL-7. Common ( $\alpha\beta^+$  TCR CTL-defined) Ags shared by tumors of different histologies have recently been reported in murine (52) and in human (53) tumors. One may speculate that such  $\gamma\delta$  CTL-defined crossreactivity may reflect similar cellular target structures associated with cells of epithelial origin. Of note, antigenically defined ( $\alpha\beta^+$ ) T cell reactivity observed in human cancer (melanoma [54] and renal cell cancer [53]) supports the concept that antitumor cellular immunity, in fact, represents an autoimmune response, a finding that may be equally applicable to antitumor-reactive  $\gamma\delta$  T cells.

CD1 has been reported to be expressed by intestinal cells (14, 55) and may serve as a potential target structure for certain  $\gamma\delta$  T cells. In mice, the nonclassical Tla Ag, also expressed in the intestine (15), may be recognized by  $CD8^+$  intestinal  $V\gamma 5^+$  T cells (15, 16). mAbs directed against MHC class I (w6/32) or II DR (mAb L243), or mAbs recognizing CD1a or CD1c were not able to block  $\gamma\delta$  T cell-mediated cytotoxicity of target cell lines (data not shown).

Whereas this suggests that  $\gamma\delta^+$  T cell recognition of autologous or allogeneic cancer cell lines is not restricted by MHC class I or II, the inability of anti-CD1 mAbs to block  $\gamma\delta$  T cell reactivity may not be informative because of inefficient blocking of relevant determinants. Examination of target cell surface expression of CD1 Ags revealed that only the cell line Molt-4, not recognized by  $V\delta 1^+$  T cells, stained positive for CD1 (data not shown).

Murine  $V\gamma 3^+$  T cells (dendritic epidermal T cells) specifically recognize keratinocytes (56). It is possible that human  $V\delta 1^+$  T cells recognize cells of epithelial origin in a tissue-specific fashion and react against target structures induced by stress (i.e., in vitro culture) and/or by malignant transformation. Potential candidates for target Ags may represent HSPs (13, 19, 20, 57). Anti-breast-cancer directed  $\gamma\delta$  T cell responses are augmented by transfecting breast tumor cells with the 27-kD HSP protein (58), suggesting a role for HSP expression in  $\gamma\delta$  T cell recognition of solid tumors. A strong correlation between cell surface expression of HSP 72 and the 67-kD high affinity laminin receptor in human lung cancer and the outgrowth of antitumor-directed cytotoxic  $\gamma\delta$  T cells (59) has also been noted. grp 75 has also been suggested to present tumor-specific Ags (presumably in the form of peptides) to  $\gamma\delta$  T cells (57). However, we were unable to inhibit T cell reactivity with a panel of anti-HSP Abs, a finding that either supports the lack of involvement of the specific Ag in  $\gamma\delta$  T cell-mediated reactivity or reflects the inability of a given mAb to block a functional epitope present on the indicated HSP (data not shown). To correlate HSP expression on individual target cells with cytolytic  $\gamma\delta^+$  T cell reactivity or specific IFN- $\gamma$  release, we examined target cell surface expression of several well-characterized HSP subsets (including HSP 60, 68, 70, 72, 73, and 75; see Table 1) by flow cytometry. In summary, all target cell lines tested negative for HSP 60, 68, 70, and 75 expression and showed variable cell surface staining using mAbs directed against HSP 72 or 73 (our unpublished observations). Target cell lines exhibiting comparably high expression of HSP 72 or 73 (e.g., Daudi, or I 937) were not recognized by  $\gamma\delta$  T cells (Fig. 3). In contrast, the human explant culture CRL7136, HS185.INT from normal human intestine lacked HSP 72/73 cell surface expression, but provided target structures recognized by  $\gamma\delta$  T cells. Based on these data, we could not identify a positive correlation of HSP 72 and 73 cell surface expression and  $\gamma\delta$  T cell recognition. However, the cell surface density of individual HSP subsets relevant for  $\gamma\delta$  T cell recognition, as well as potential peptides presented by such alternate Ag-presenting molecules, are poorly defined. A more detailed examination of individual intracellular and cell surface family HSP protein expression in targets recognized by human intestinal  $\gamma\delta$  T cells may aid clarification of the role of HSPs as candidate target structures.

Of note, four mAbs were able to partially block  $\gamma\delta$  T cell recognition of sensitive target cells: Abs directed against the TCR, against the  $\beta 2$  or  $\beta 7$  integrin chains, or an Ab against the HFR receptor. Cellular interactions with fibro-

nectin have been shown to induce phosphorylation of a 120-kD protein on T cells and HFR-mediated "costimulation" may augment T cell cytotoxicity induced by TCR stimulation (60). Fibronectin-associated T cell stimulation may therefore facilitate TCR restricted effector cell functions (e.g., granzyme release), or it may lower the signal threshold required for optimal T cell stimulation (60, 61). Blockade of the fibronectin receptor on  $\gamma\delta$  T cells may therefore interfere with  $\gamma\delta$  T cell stimulation. Fibronectin also appears to be involved in  $\alpha 4/\beta 7$ -mediated adhesion of T cells to the vascular cell adhesion molecule 1 (VCAM-1), or to mucosal addressin cell adhesion molecule 1 (MADCAM-1) (62). Interaction of  $\beta 7$  with fibronectin, MADCAM-1, and VCAM-1 involves different, functionally distinct determinants on the  $\beta 7$  integrin molecule (62). Inhibition of cytotoxic  $\gamma\delta$  T cell responses with an Ab directed against the  $\beta 7$  integrin chain may reflect the importance of  $\beta 7$  integrin ligands (i.e., MADCAM, fibronectin, etc.) expressed by tumor cells of epithelial origin.

It should be noted that differences in  $\gamma\delta$  T cell responses to individual targets as measured by cytotoxicity and IFN- $\gamma$  release may be attributed to the presence of more than one discrete effector T cell population as defined by differential cell surface expression of CD8 and CD4 (Fig. 2). CD8 appears to be preferentially expressed on V $\delta 1^+$  T cells, predominantly in the form of a CD8  $\alpha$  chain homodimer, which has been shown to be inducible by various cytokines (e.g., IL-4; 63–65). The effects of IL-1 $\beta$  or IL-7 cell surface expression on CD8 chains has not been evaluated to this end and awaits further clarification. CD8 acts as an adhesion molecule for  $\alpha\beta$  TCR $^+$  T cells and as a signal-transducing molecule. The function of CD8 on intestinal  $\gamma\delta$  T cells remains elusive, since: (a) CD8 expression appears to be rapidly induced after tissue homing, suggesting that CD8 expression may not be correlated with conventional Ag-driven activation occurring in MHC class I-restricted  $\alpha\beta^+$  T cells (66); and (b) target cell recognition by V $\delta 1^+$  T cell clones appears not to be affected by CD8 blockade (67). Additionally, direct cloning of freshly isolated CD4 $^+$  TCR  $\gamma\delta^+$  T cells indicated that V $\delta 1^+$  T cells may also express CD4 in vivo. It is interesting to note that such CD4 $^+$   $\gamma\delta$  T cells have been characterized to lack cytolytic functions, but to secrete high levels of cytokines (65). These data may suggest that variations in  $\gamma\delta$  T cells responses (e.g., different levels of inhibition of target cell lysis using mAbs directed against the  $\gamma\delta$  TCR; Fig. 4) may reflect the presence of such distinct  $\gamma\delta$  T cell subpopulations defined by CD8 and CD4 expression, correlating with different (i.e., cytotoxicity and cytokine release) T cell effector functions.

Partial inhibition of target cell recognition by Abs directed against the  $\gamma\delta$  TCR, indicates that cytolytic V $\delta 1^+$  T

cell responses are mediated by specific target cell recognition involving the TCR. Potential ligands for  $\gamma\delta$  TCR recognition may represent native target cell surface structures (11, 12, 17, 19, 20, 21, 68), consolidating the notion that the  $\gamma\delta$  T cell population may use a fundamentally different method of target cell recognition as compared with that of the MHC-restricted  $\alpha\beta$  T cell population. Significant IFN- $\gamma$  release by three  $\gamma\delta$  TIL lines derived from colon cancer patients in response to autologous or allogeneic colon cancer cell lines suggests that these  $\gamma\delta$  T cells may be therapeutically important.  $\gamma\delta$  T cells may therefore not only be useful as effector T cell subsets capable of directly eradicating cancer cells when adoptively transferred into cancer patients, but they also may be useful in creating a "Th1-like" cytokine milieu within the tumor lesion microenvironment. IFN- $\gamma$  may act directly on tumor cells e.g., by, for example, increasing cellularly expressed MHC products, which serve as restricting molecules for MHC-presented peptides capable of being recognized by  $\alpha\beta^+$  T cells. IFN- $\gamma$  release in response to cancer may be particularly important in the case where tumor cells display resistance to perforin-mediated lysis (e.g., HT29; Fig. 3), which may reflect tumor cell overexpression of CD59, a molecule capable of preventing perforin complex formation into lytic structures on the target cell surface (69). The intimate interaction of  $\gamma\delta$  and  $\alpha\beta$  T cells has previously been documented and it is envisioned that  $\gamma\delta^+$  T cells may impact significantly upon the quality and magnitude of  $\alpha\beta$  T cell-mediated immune responses (70–74).

In summary, our results demonstrate that  $\gamma\delta$  TIL lines obtained from colon cancer recognize target structures expressed by colon, pancreatic, and by renal cell cancer cells in a MHC-unrestricted manner. This particular recognition pattern is shared by  $\gamma\delta$  TIL lines obtained from three different patients, indicating that the prevalent V $\delta 1^+$  TCR subpopulation may play a role in anti-tumor-directed immunity. Examination of a limited number of TCR transcripts from colon cancer-derived  $\gamma\delta$  T cell populations expanded in vitro supports the preferential usage of the V $\delta 1$ /D $\delta 3$ /J $\delta 1$  TCR segments that have been reported to represent common  $\gamma\delta$  TCR transcripts observed in colon biopsies harvested from normal healthy individuals (5–8). The junctional TCR sequences in  $\gamma\delta$  TIL appear to be diverse, although there was some common usage of certain amino acid residues in the N/P regions shared by individual cDNA clones. We believe that the exclusive use of TCR V $\delta 1$  does not reflect a preferential homing receptor for human intestinal  $\gamma\delta$  T cells, but rather reflects the inherent useful nature of this particular VDJ recombination in TCR reactive against certain Ags expressed by stressed intestinal cells or by epithelial cancer cells.

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